THE UNIVERSITY OF ALBERTA

Nitrogen Fixation in *Shepherdia canadensis* (L.) Nutt.

by

© Janet Elizabeth McLean

A THESIS

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SHEPHERDIA
CANADENSIS

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Nitrogen Fixation in *Shepherdia canadensis* (L.) Nutt. submitted by Janet Elizabeth McLean in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Shepherdia canadensis (L.) Nutt. seedlings were reared in a controlled environment 'growth room' for 3.5 months. They were then outplanted onto a simulated reclamation site north of Fort McMurray, Alberta. Seedlings were supplied with 4 mg, 35 mg, or no nitrogen over the growth room period. The peat rooting medium was injected with a *Frankia* (EUN1f) solution at 5 or 9 weeks after germination and control seedlings received only nitrogen-free Crone's solution. Nodulation and seedling growth and survival were observed after the growth room period (3.5 months after germination) and 2.5 months after outplanting (6 months after seedling germination).

Functional nodules developed only on *Shepherdia* seedlings which were inoculated with *Frankia*. Fewer plants became nodulated at the second inoculation than at the first but more nodules were produced per seedling at the second inoculation. The level of nitrogen that seedlings received did not significantly affect whether or not seedlings became nodulated, and inoculated seedlings receiving 35 mg of nitrogen produced the largest total weight of nodules. After outplanting, virtually all seedlings became nodulated but inoculated seedlings produced a larger total weight of nodules per seedling than uninoculated seedlings. Again, seedlings supplied with 35 mg of nitrogen before outplanting had the greatest weight of nodules per seedling.

Inoculated seedlings produced the greatest shoot and root weights before and after outplanting in all nitrogen treatments although differences between inoculated and uninoculated seedlings receiving 35 mg of nitrogen were not statistically significant. Seedling survival over the entire growth room and outplanting periods appeared to be enhanced by inoculation in the 0 mg and 4 mg nitrogen treatments.

The contribution of *Shepherdia* to soil nitrogen was also investigated on one natural forest site north of Fort McMurray, Alberta. *Shepherdia*'s leaf litter was very nitrogen rich at leaf senescence (1.6% N) and *Shepherdia* leaves decomposed more rapidly than the leaves of *Populus tremuloides* (a dominant source of leaf litter on the study site). Samples of the FH layer beneath *Shepherdia* were incubated at 28° C at moisture levels near field capacity and a large amount of ammonium (about 500 ppm) was mineralized from samples over a three week period. Although *Shepherdia* was widespread over the study site, its litter was restricted to about a 1.5 m radius around shrub bases and incorporation of litter into mineral soil was minimal.

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LIST OF ABBREVIATIONS

N	nitrogen
P	phosphorous
K	potassium
S	sulphur
C	carbon
N -	refers to the amount of nitrogen seedlings received (see individual Methods sections)
N +	same as N -
Full N	same as N -
P/V	a rooting medium made up of 2 parts peat to 1 part vermiculite (by volume)
V	a rooting medium consisting only of a horticultural grade of vermiculite
S	a rooting medium made up of 1 part peat to 1 part vermiculite to 1 part sand (by volume)
I -	no *Frankia* inoculum supplied
I (1)	*Frankia* inoculum supplied to seedlings 5 weeks after germination (also indicated as (I 1)
I (2)	*Frankia* inoculum supplied to seedlings 9 weeks after germination (also indicated as (I 2)
nod	nodule
nod -	seedlings with no nodules
nod +	seedlings with nodules

fix	fixation (in terms of acetylene reduction)
s.d.	standard deviation
APL	A Programming Language
SPSS	Statistical Package for the Social Sciences
ANOVA	analysis of variance
df	degrees of freedom
MS	mean square
F	Fisher's F value
sig	significance
*	significant at the .05 alpha level
NS	not significant at the .05 level
Tukey HSD	Tukey's honestly significant difference
e.c.	electrical conductivity
LFH	the organic layer at the soil surface which contains recent litter, decomposed litter, and well-humified organic matter
FH	does not include the recent leaf litter of the LFH layer
o.d.	oven dry

I. INTRODUCTION

Shepherdia canadensis (L.) Nutt. (hereafter referred to by its generic name only) is a wide-ranging shrub occurring in North America from arctic Canada south into areas of Maine, New York, Oregon, and along the Rocky Mountains into New Mexico (Viereck and Little 1973). In Alberta, *Shepherdia* is common on slopes of the eastern Rocky Mountains and in spruce or pine vegetation associations of the boreal forest (Corns 1978; Krumlick *et al.* 1978; Krumlick *et al.* 1979). The species has been present in Alberta since immediately after glaciation or about 11,000 years (Lichti-Federovich 1970) and its presence is considered to be an indication of poor soil fertility (Moore 1964; Moss 1953).

Shepherdia commonly forms large nodules on its roots (Allen *et al.* 1964; Moore 1964) and these nodules have been demonstrated to fix nitrogen (Bond 1957; Gardner and Bond 1957). Several other non-leguminous angiosperms also have the ability to fix nitrogen symbiotically, and the organism inhabiting all of these root nodules is thought to be an actinomycete (Becking 1970; Bond 1977; Lechavalier and Lechavalier 1979). In 1978, the actinomycete-like organism which participates in the nitrogen-fixing symbiosis in non-legumes was cultured outside of root nodules (Berry and Torrey 1979; Lalonde and Calvert 1979). Scientists had been attempting to culture the organism for 80 years and the final success was an important breakthrough in the study of symbiotic nitrogen fixation (Baker and Torrey 1979). It is

possible that actinomycete cultures (artificial inocula) will soon become commercially available and inoculation of nitrogen-fixing non-legumes will become feasible on an operational scale. Artificial inoculation would enable symbiotic non-legumes to develop nodules in soils where native inoculum is absent or of poor quality. Reclamation sites are an important case where the abundance of native inoculum is not assured.

Reclamation scientists involved with oil-sand mining in northeastern Alberta are presently interested in *Shepherdia* as a reclamation species (Fessenden 1979). Preliminary studies assessing the potential of *Shepherdia* as a reclamation species have been encouraging: the species appears to be adapted to the environmental conditions of reclamation sites (Berry and Klymm 1974; Fedkenheuer 1979). *Shepherdia* seedlings are reared in greenhouses for a three to six month period before they are outplanted and an artificial inoculum could be introduced to seedlings during this period if nodulation enhances *Shepherdia*'s outplanting success. It is important to assess the benefits that artificial inoculation can provide to *Shepherdia* now before large-scale outplantings of the species begin.

Along with concerns about the success of *Shepherdia* on reclamation sites is a concern for how important the nitrogen-fixing abilities of *Shepherdia* are to the nitrogen economy of wildland soils. It is of interest to reclamation scientists to assess whether *Shepherdia* can raise soil

nitrogen to levels that may significantly benefit other plants. The objective of reclamation is to bring land back to its original, or better, productivity (The Resource Handbook 1979) and, because reclamation sites created by oil-sand mining activities are nutrient poor, the most ecologically and economically desirable means of achieving this objective is to reclaim areas with vegetation associations that are self-sustaining. Viereck (1966) has already indicated that *Shepherdia* may be an important successional species because of its role in soil development. Again it is important to assess *Shepherdia*'s contribution to soil nitrogen as soon as possible so that future outplantings can be designed to provide maximum nitrogen benefits.

Nodule induction and soil nitrogen effects are two related but distinct topics and although a thorough analysis of either could provide enough information for several studies, it is advantageous to consider the topics together to facilitate the management of *Shepherdia* on reclamation sites. It is the overall objective of this thesis to provide a broad information base for future research while addressing some specific concerns for the management and utilization of *Shepherdia*.

This thesis can be divided into two studies each with its own objectives. The general objectives of Study I concern nodulation and they are as follows:

a. To determine whether an artificial inoculum can induce nodules on *Shepherdia* seedlings reared under greenhouse procedures which are operationally feasible.

b. To assess whether greenhouse inoculation affects the survival and growth of *Shepherdia* seedlings after outplanting.

Study II deals with some aspects of soil nitrogen and its objective is as follows:

To examine *Shepherdia*'s potential contribution to available soil nitrogen through leaf fall.

The two studies are dealt with separately in this thesis.

II. STUDY I. NODULATION

The first study was designed to answer the following questions:

1. Can an artificial inoculum be used to induce nodules on greenhouse-reared *Shepherdia* seedlings?

   and

2. Will inoculation before outplanting affect seedling survival and growth after outplanting?

However, before an artificial inoculum can be tested, some preliminary information must be collected. A rearing regime must be designed to ensure that host plants are growing well enough for nodulation to occur and for nodules to function properly. It is also necessary to establish how much nodulation can occur without an artificial inoculum —inoculation will not be necessary if nodulation occurs readily without a supplied inoculum.

Three experiments were designed to provide basic information about *Shepherdia*'s growth and nodulation before artificial inoculum tests were performed. These experiments are collectively referred to as the Initial Growth and Nodulation Experiments. The first experiment, The Preliminary Growth Experiment, was designed to gain familiarity with the rearing and development of *Shepherdia* in three different rooting media with three different nitrogen applications and to describe nodules induced by a crushed-nodule inoculum. The second experiment, The Fertilizer Experiment, performed in conjunction with the

Preliminary Growth Experiment to examine whether the amounts of the four macro-nutrients (nitrogen, phosphorous, potassium, and sulphur) applied to seedlings in the Preliminary Growth Experiment were adequate for the proper growth and development of *Shepherdia* seedlings. This experiment was supplemental to the Preliminary Growth Experiment serving only as a 'double-check' to ensure that seedlings reared for artificial inoculum tests would receive adequate supplies of all macro-nutrients. The specific methods and the results of the Fertilizer Experiment are included in Appendix v, but an overview of the experiment has been included in the text of the thesis. The third experiment, The Peat Experiment, was designed to determine how much nodulation can occur in various peat sources without any introduced inoculum.

After a growing regime for *Shepherdia* had been tested and refined in the Preliminary Growth and Nodulation Experiments, the Artificial Inoculum Experiment was designed. This experiment was the focus of Study I— the previous experiments served only to refine growing techniques, or provide information necessary for the interpretation of results obtained in artificial inoculum trials.

III. METHODS: STUDY I

Before individual experiments are described, general rearing procedures common to all experiments will be outlined. These procedures did not change greatly from one experiment to the next and including them together saves later repetition. The general rearing procedures outlined cover both the Initial Growth and Nodulation Experiments and the Artificial Inoculum Experiment.

General Rearing Procedures

1. Seed

   a) source: seed was obtained from sites north of Fort McMurray, Alberta:
   approximate latitude 56° 39′ North
   approximate longitude 111° 13′ West
   approximate elevation 370 m (above sea level)

   b) preparation: the seed was cold-stratified between layers of moist vermiculite at 1°C for 3 months. All containers were planted with pre-ferminated seeds to ensure a very narrow age range among seedlings.

2. Rooting volumes and Media

-Spencer-Lemaire Hillson size (172 cm^3) planting containers were used for all experiments. These containers consist of four planting cavities joined together to form a row (book).
The surface area of planting cavities was calculated to convert nutrient applications to kg/ha equivalents. Each planting cavity has a surface area of approximately 1.452×10^{-7} ha.

-Unless otherwise specified, the rooting medium used for experiments was Alberta Rose peat (a commercial peat manufactured in Alberta) which was mixed in a 2/1 ratio with a horticultural grade of vermiculite.

-Unless otherwise specified, seedlings were planted in every other planting cavity with intervening cavities left empty.

-Rooting media were kept constantly moist.

3. Fertilizer application

-Each fertilizer dose was mixed with city of Edmonton tap water and dispensed with a Brinkman Pipetter. Fertilizer solutions were applied to seedlings beginning four weeks after germination. Total fertilizer amounts were distributed over several doses. About 15-20 mls of fertilizer solution were applied to seedlings at each application.

4. The growing regime for each experiment was as follows:

	Preliminary and Fertilizer Expts.	Peat and Artificial Inoculum Expts.
Watering:	automatic	by hand
RH:	65%	75 to 100 %
Light:		
system	incandescent with natural	fluorescent with 10% incandescent
intensity	1000 to 2000	2000 ft c
day length	20 hrs	17 hrs
Temperature:		
day	23 to 28°C	17 to 21°C
night	18°C	same as day

ANALYSIS PROCEDURES

Analysis procedures also did not vary from one experiment to the next. Any analysis procedure used in two or more experiments has been included below.

1. Plant weights

   All plant material was oven dried at 60° C for 24 hrs to obtain oven-dry weights.

2. Acetylene reduction

   Roots were cut at the root collar, washed, and lightly blotted to remove excess water. They were then put into 40 ml serum bottles and stoppered with serum stoppers. 5 mls of air were evacuated from each bottle and replaced with 5 mls of acetylene. Roots were incubated for a total of 10 hours with 1 ml samples of gas removed at

2 hours and at 10 hours to check for ethylene (Hardy *et al*. 1968; Hardy *et al*. 1973; Silver 1969). The analysis was carried out on a Beckman GC 4 gas chromatograph with a hydrogen flame detector and a Columbia Scientific Industries' Supergrator-1 was used to measure attenuation. No extrapolations from ethylene produced to nitrogen fixed were planned because of the variability in the relationship between reduction of acetylene and the reduction of dinitrogen (Bergersen 1970; Sprent 1979).

3. Nitrogen concentrations

Tissue samples were crushed to pass through a 500 micron sieve. (Approximately .5 g of tissue were collected for each sample.) Samples were put through a micro-Kjeldahl digestion (no inclusion of NO3-) and steam distillation (McKeague 1978). All nitrogen concentrations were based on oven-dry weights.

4. Experimental designs and data analyses

Experiments were designed to facilitate statistical analyses by the analysis of variance technique (ANOVA), a standard technique used for the analysis of factorial experiments (Steel and Torrie 1980). The alpha level selected to decide whether differences were statistically significant was .05. This level was chosen because it meets the standards of scientific

journals without being so stringent that the chance of a Type II error is prohibitive. (There was very little information about rearing *Shepherdia* when experiments were begun, and experimental error was expected to be high, increasing the chance of Type II errors.) For all ANOVAS performed, it was assumed that data met the underlying assumptions required for valid testing by the ANOVA technique. Percentage data were treated the same as other data and not transformed logarithmically. This was done under the assumption that incompatabilities of these data with the underlying assumptions of ANOVA testing were unimportant to the overall assessment of treatment factors analyzed (Steel and Torrie 1980). The ANOVA has been included in the thesis for the reader's own scrutiny whenever percentage data were analyzed.

A. PRELIMINARY GROWTH EXPERIMENT

OVERVIEW

Exact prescriptions for the optimal rearing of *Shepherdia* seedlings are not available but general practices used for growing containerized seedlings have been successfully applied to *Shepherdia* (A. Fedkenheuer pers. comm.). However, because this experiment has been designed to refine a growing regime for use in artificial inoculum tests, particular attention must be given to a number of factors affecting nodulation and nitrogen fixation. These factors include moisture, temperature, light, soil fertility, amount of available nitrogen, and soil pH (Perry *et al.* 1979; Wheeler and McLaughlin 1979). Nutrient levels, especially nitrogen, are critical to the nodulation and fixation processes (Bond and MacKintosh 1975; Rodriguez-Barrueco *et al.* 1970; Stewart and Bond 1961; Zavitkovski and Newton 1968). Nutrient levels for *Shepherdia* can be formulated from general information about plant requirements, and nutrients specific to nitrogen-fixing systems can be added to general nutrient mixes. Nitrogen applications must be varied so that inocula can be tested on seedlings with varying degrees of nitrogen deficiency, and this necessitates experimentation to select nitrogen levels that will induce variability without damaging seedlings.

Containerized seedlings presently reared for oil-sand reclamation are grown in peat or peat/vermiculite soil media. However, most experiments investigating nodulation in

non-legumes have made use of hydroponic or very artificial soil-like media for rearing seedlings. The medium in which a containerized seedling is grown can affect root egress from planting containers into soil after outplanting (A.K. Hellum pers.comm.) and this could be significant with regard to outplanting success and nodulation after outplanting. Information about *Shepherdia*'s growth in a peat/vermiculite medium relative to some other soil-like medium is necessary so that the growth of seedlings in future experiments and outplanting trials can be optimized.

Historically, greenhouse nodulation in non-leguminous angiosperms has been induced by inoculation with crushed nodules or habitat soils (Gardner and Bond 1957; Stewart and Bond 1961; Rodriguez-Barrueco *et al*. 1970). A crushed-nodule inoculum is not an operationally feasible means of nodulating seedlings but nodulation resulting from this inoculum source should be examined so that comparisons with nodules resulting from an artificial inoculum can be made.

The objectives of the Preliminary Growth Experiment were as follows:

1. To investigate seedling growth and nitrogen uptake in three nitrogen levels to assist with the design of future experiments.
2. To investigate the growth of seedlings in three different soil media to assist with the design of future experiments.

3. To obtain information about the growth and development schedule of *Shepherdia* for the design of future experiments and to become familiar with the rearing of *Shepherdia* so that plants in future experiments would be healthy and vigorous.
4. To test the nodulation potential of a crushed-nodule inoculum on seedlings for future comparisons with an artificial inoculum.

In order to satisfy the above objectives, growth curves for *Shepherdia* were obtained along with the following information:

a. shoot and root weights, and shoot/root ratios of seedlings grown in the different media and nitrogen treatments, and
b. the concentration of nitrogen in leaves, roots, and stems of seedlings reared in the three different nitrogen treatments.

TECHNICAL DETAILS

1. Duration:

   September 21 to December 27, 1979

2. Seedlot:

   seed was from a bulked collection obtained in summer 1978

3. Nutrients:

   Total non-treatment nutrients were supplied as follows (kg/ha equivalents are based on the surface area of seedling containers):

Per Seedling Application Rates

NUTRIENT	AMOUNT (mg)	AMOUNT (kg/ha)
P	56	386
K	52	358
S	11	73

The following micro nutrients were also supplied:

NUTRIENT	AMOUNT (micro g)
B	102
Mn	108
Zn	12
Mo	3.5
Cu	4.4
Co	10.8

(see Appendix i for the chemical sources of each nutrient).

Iron was supplied in the form of a commercial iron chelate (Plant-Green Iron Chelate manufactured by Plant Products Company Ltd. Bramalea, Ontario). A total of .05 g/seedling

was applied.

4. Experimental design:

A three factor factorial experiment arranged as a randomized complete block.

5. Treatments:

a)3 growth media:

(P/V) Peat/Vermiculite (2:1)

(V) Vermiculite alone

(S) Peat/Sand/Vermiculite (1:1:1)

(the sand was obtained from the Syncrude Pilot plant and was similar to processed oil sand)

b) 3 Nitrogen levels:

	AMOUNT (mg)	kg/ha equivalent
N-	0	0
N+	5	37
Full N	56	386

c) 2 Inocula:

(I+) crushed nodules applied in a water slurry at six weeks

(I-) no crushed nodule applied

The experiment was arranged into five blocks along the greenhouse bench to adjust for variability in growing conditions.

6. Experimental procedures:

a)inoculation:

A crushed nodule slurry was prepared from air-dried nodules collected about 10 km east of the Syncrude plant-site near Fort McMurray, Alberta (approximately same latitude, longitude, and elevation as seed collections) The nodules were crushed with a mortar and pestal and added to distilled water to produce a concentration of about 100 mg of nodule per 15 mls of water. The inoculum was then watered onto seedlings from a graduated cylinder (15 mls per seedling).

b)fertilizer:

Approximately equal nutrient increments were supplied at each application.

7. Experiment Dimensions:

3 seedlings (repeats) were randomized within each unique treatment combination: (3 growth media X 3 N levels X 2 inocula X 2 repeats X 5 blocks)= 270 seedlings in total.

8. Analysis:

-repeats were pooled providing one mean observation for each unique treatment combination

-data were analyzed using the APL packages AOV5 for ANOVAS, MEANS to obtain means, and OC for orthogonal comparisons.

B. FERTILIZER EXPERIMENT

OVERVIEW

As was mentioned earlier, the Fertilizer Experiment was supplemental to the Preliminary Growth Experiment. Its only purpose was to ensure that amounts of macro-nutrients applied to seedlings in the Preliminary Growth Experiment were adequate for the proper growth and development of *Shepherdia* seedlings. Although only nutrient levels were of interest in this experiment, two rooting media were included as experimental treatments in case responses to nutrients varied with soil media (because this experiment was conducted concurrently with the Preliminary Growth Experiment, it was not yet known which medium would be selected for future experiments). The objective of the Fertilizer Experiment was to answer the following question:

How much nitrogen, phosphorous, potassium, and sulphur must be supplied to seedlings before they cease to show a response to further additions?

In order to answer the above question shoot and root weights of seedlings reared with different levels of N,P,K, and S were measured.

The technical details of this experiment and the results are contained in Appendix v.

C. PEAT INOCULUM EXPERIMENT

OVERVIEW

The Peat Inoculum Experiment was designed to determine the extent to which nodulation can occur in various peat sources without a supplied inoculum. The primary objective of this experiment was to answer the following question:

Can nodules occur on seedlings reared in a peat medium without a supplied inoculum?

The second objective, arising from the primary objective, was to characterize any nodules induced with respect to number per seedling, biomass (the total weight of nodules per seedling), and fixation per seedling, and to document the effects of these nodules on plant growth. The final objective of this experiment was to compare the growth of seedlings in various peat media in order to assess whether Alberta Rose Peat, the peat used in all experiments, promoted good seedling growth relative to other peats available for use. Alberta Rose had been pre-selected for experiments because its source and processing were known and because the peat was very homogeneous. Comparing the growth of *Shepherdia* in Alberta Rose to growth in other peats ensured that if some gross factor were affecting *Shepherdia* seedlings in Alberta Rose peat, the influence on experimental results would not be overlooked.

In order to satisfy the above objectives, the following responses were recorded:

a. the occurrence of nodulated seedlings in the different

peat media,

b. the average number of nodules, nodule biomass, and fixation levels produced per seedling in the different peat media,

c. the effect of nodules on seedling shoot and root growth, and

d. shoot and root weights, and shoot/root ratios of non-nodulated seedlings grown in the different peat media.

TECHNICAL DETAILS

1. Duration:

   February 8 to June 2, 1980.

2. Seedlot:

   one seedlot collected in the summer of 1979

3. Nutrients:

   Total non-nitrogen nutrient dosages were as follows:

   (kg/ha equivalent is based on container surface area)

	per seedling application rates	
nutrient	dose (mg)	kg/ha
P	12	85

micro nutrients	micro g
B	97
Mn	103
Zn	12
Mo	3
Cu	4
Co	10

Fe Chelate .02 g/seedling

4. Experimental design:

A completely randomized two factor factorial experiment.

5. Treatments:

a)five peats:

-native: an unprocessed, undried peat from a source area near Ft. McMurray

-Sunshine (old): a commercial peat in a bag that had been opened for some time

-Sunshine (new): the same peat brand as above but the bag had been recently purchased for this experiment

-Parkland: a commercial peat produced by Langley Peat

-Alberta Rose: the commercial peat that was used in all the other experiments

b)three nitrogen levels:

(kg/ha equivalents are based on container surface areas)

	per seedling application rates	
	mg	kg/ha
N-	0	0
N+	4	27
Full N	41	280

6. Experimental procedures:

a)peats were mixed with a horticultural grade vermiculite in a peat:vermiculite (2:1) ratio

b)fertilizer was applied in increasing amounts as growth increased (*i.e.* as seedling nutrient requirements increased)

7. Experimental dimensions:

8 rows of seedlings (randomized) for each unique treatment combination with each row containing 2 seedlings (repeats)

(5 peats X 3 N levels X 8 rows X 2 repeats/row) = 240 seedlings in total.

8. Analysis:

-repeats were pooled to provide 8 mean observations for each unique treatment combination

-data were analyzed using the APL packages AOV5 for ANOVAS and AMEH.MCTEST for multiple comparisons

D. ARTIFICIAL INOCULUM EXPERIMENT

The objectives of the Artificial Inoculum Experiment were first, to determine whether an artificial inoculum could induce nodules on greenhouse-reared *Shepherdia* seedlings and second, to determine if inoculation before outplanting affected seedling survival and growth after outplanting. Although only one experiment was designed to satisfy the above objectives, observations were separated in space and time requiring that the experiment be divided into two sections— a growth room rearing period and an outplanting period.

GROWTH ROOM: OVERVIEW

Inoculation was conducted in a growth room instead of a greenhouse to eliminate the large fluctuations in light and temperature encountered in greenhouses. This 'growth room rearing period' was to answer the following question:

To what extent will *Shepherdia* seedlings receiving an artificial inoculum nodulate?

Nodules induced by an artificial inoculum would provide general information about the nodules of non-leguminous nitrogen-fixers and the second objective of the growth room rearing period was to characterize any nodules induced by the artificial inoculum with respect to number of nodules per seedling, biomass (total weight of nodules per seedling), and fixation per seedling. The third objective was to identify the effects of inoculation on the growth of *Shepherdia* seedlings to assist with the interpretation of

outplanting information. If survival is affected by inoculation, it will be necessary to determine why this happens so that results can be generalized to other outplanting situations.

The following information was recorded in this experiment:

a. the proportion of nodulated seedlings in the different inoculum and nitrogen treatments,
b. the average number, biomass, size, and fixation level of nodules induced by the artificial inoculum,
c. the correlations among nodule characteristics,
d. nodule efficiency in terms of micromoles of ethylene fixed per hour per gram of nodule (fresh weight),
e. shoot and root weights, and shoot/root ratios of seedlings reared in the different inoculum and nitrogen treatments,
f. correlations between shoot and root weights and nodule biomass, and
g. average nitrogen concentrations and contents in leaves, roots, and stems of non-nodulated and nodulated seedlings reared in different nitrogen treatments.

GROWTH ROOM: TECHNICAL DETAILS

1. Duration:

   February 28 to June 9, 1980

2. Seedlot:

   same as the Peat Experiment

3. Nutrients:

   Total non-nitrogen doses were as follows:

   (kg/ha estimate based on container surface area)

   per seedling application rates

Phosphorous	11 mg (73 kg/ha)

micro nutrients	(micrograms)
B	73
Mn	77
Zn	9
Mo	2
Cu	3
Co	8

4. Experimental design:

   A two factor factorial experiment arranged as a randomized complete block.

5. Treatments:

   a)Three inocula:

   (I-) Nitrogen free Crones solution alone

   (I 1) Nitrogen free Crones solution with inoculum injected at 5 weeks after germination (approximately 4-6 leaf stage)

(I 2) Nitrogen free Crones solution with inoculum injected at 9 weeks after germination (approximately 10 leaf stage)

b) Three Nitrogen levels:

application rates per seedling

	Total mg	Total kg/ha
N-	0	0
N+	4	29
Full N	35	244

(kg/ha equivalents are based on container surface areas)

Proportion of nitrogen supplied

	at I (1)	at I (2)
N-	*	*
N+	24%	50%
Full N	15%	41%

The experiment was arranged into forty-eight blocks along the growth room bench to adjust for variability in growth conditions.

6. Experimental Procedures:

a) preparation of nitrogen free Crones solution

KCl	10.0 g
CaSO4	2.5
MgSO4	2.5
(Ca)3PO4	2.5
FePO4	2.5

These salts were ground together and 1.5 g of the mixture was dissolved in 1 litre of distilled water.

b)application of inoculum:

Vacutainer tubes containing pelleted *Frankia* (strain EUN1f) in N-free Crone's solution were shipped from Dr. Maurice Lalonde at the Charles F. Kettering Institute at Yellow Springs, Ohio. *Frankia* cells were resuspended into the Crone's solution by repeated 'flushing' of cells through a syringe. Vial contents were then added to 250 ml erlenmeyer flasks containing 200 mls of N-free Crone's solution (prepared as above). The *Frankia* solution was kept homogenized with a magnetic stirrer apparatus. Inoculum was applied to seedlings in 2 ml aliquots with a Cornwall constant pipettor syringe. Inoculum was injected into the peat near seedling root crowns in order to distribute inoculum into the rhizosphere of seedlings. For the first inoculum, an 18 G 1.5 syringe was used; for the second inoculum, a dispensing syringe was used. Inoculations were performed in sequence from one bench end to the other for convenience and to insure that no seedlings would be missed.

No fertilizer was administered to seedlings 10 days before or after inoculation and for 2 weeks before acetylene reduction was tested.

c)fertilizer:

Amounts administered at each application were increased as plant growth increased

7. Experiment Dimensions:

1 container row per each unique treatment combination with 2 seedlings(repeats) per container row.

(3 N levels X 3 inocula X 2 repeats X 48 blocks)= 864 seedlings in total.

8. Analysis:

-repeats were pooled providing one mean observation per unique treatment combination although, for some of the nodule analyses, repeats were not pooled. Analyses that did not use pooled data are identified in the RESULTS section.

-data were analyzed using the SPSS packages ANOVA for Means and ANOVAS and the APL programs AOV5 for nodule ANOVAS, (AMEH.MCTEST) for multiple comparisons, and REG for regressions.

OUTPLANTING: OVERVIEW

Seedlings were outplanted after the growth room rearing period to assess whether growth room inoculum treatments enhanced survival or growth after outplanting. The objectives of the outplanting period were as follows:

1. To determine how much nodulation can occur naturally after outplanting.
2. To determine how growth room nitrogen and inoculation treatments affect nodule characteristics after outplanting.
3. To identify the effects of inoculation on the growth of *Shepherdia* seedlings after outplanting.
4. To determine how nitrogen and inoculum treatments affect survival after outplanting.

Three treatments were imposed on seedlings at outplanting, but these treatments were only to ensure that some seedlings would survive over the first growing season. Although the effects of these treatments on survival were analyzed, this was not considered an objective of the outplanting trial.

Only one growing season was available before analyses were performed, but sufficient seedlings were planted for excavations to continue over two more years under the supervision of Syncrude Canada Ltd. The following information was collected after the first outplanting season:

a. the occurrence of nodulated seedlings in the different inoculum and nitrogen treatments,
b. the average number and biomass of nodules on seedlings and the net increases in nodule number and biomass over the outplanting period,
c. shoot and root weights of seedlings in the different inoculum and nitrogen treatments and the net increases in those weights over the outplanting period, and
d. the proportion of seedlings surviving in the different nitrogen and inoculum treatments.

OUTPLANTING: TECHNICAL DETAILS

1. Duration:
   June 23 to September 9, 1980
2. Description of the outplanting site:
   Seedlings were planted in the Alberta Forest Service reclamation research plot near Fort McMurray. Peat and clay-textured overburden had been added to native sand within the research plot to simulate an actual tailing sand reclamation site. The plot was fenced to minimize rodent damage.

A soil analysis provided the following site information:
(Note:ratings are for agricultural soils)

total N	(.4-.5)%
pH	7.8
e.c.	.8
sodium	low
calcium	low
organic matter	low
texture	clay/loam

3. Experimental design:
   Split plot design.
4. Treatments:
   The three nitrogen treatments and the three inoculum treatments from the growth room period were already imposed on outplanting blocks.
   Upon outplanting, seedling blocks were divided into three groups at random and each group received an 'Outplanting Treatment'. Seedlings were either watered, clipped, or left as they were.

There were 39 blocks of seedlings outplanted with 13 blocks per outplanting treatment.

5. Experimental Procedures:

a)clipping- stems were clipped to remove about half of live leaves which amounted to approximately half the shoot length in order to reduce transpiration.

At outplanting seedling heights were as follows:

TREATMENT	ht (cm)	s.d.
clipped	10	3
other	22	5

b)watering- seedlings were watered with 200 mls of Ft. McMurray tap water both in the morning after outplanting and that same evening. Seedlings were also watered one month later in mid July (200 mls per seedling).

6. Experiment dimensions:

At outplanting there were 13 rows of seedlings per unique treatment combination. Seedling rows contained up to 2 seedlings per row but some contained only 1 seedling due to mortality (in a few cases both seedlings within a planting container were missing).

(3 N levels X 3 inocula X 2 repeats X 39 blocks)= 702 seedlings (approximately).

Seedlings were planted in the same row arrangements they had in the greenhouse.

7. Analysis:

-3 blocks from each outplanting treatment were selected at random and removed from the outplanting experiment for analysis. Repeats were not pooled.

-data were analyzed by the programs AMEH:desmat, AMEH:lsqanova (missing data handled by a regression approach), and AOV5 for ANOVAS. SPSS:REGRESSION was used for regressions within Nitrogen treatments. NOTE:sampling error was determined from computations using the SPSS program ANOVA . Experimental error was calculated as total error (from AMEH:lsqanova) minus sampling error.

IV. RESULTS: STUDY I

A. INITIAL GROWTH AND NODULATION EXPERIMENTS

PRELIMINARY GROWTH EXPERIMENT

Growth and development curves for *Shepherdia* over the three month rearing period are presented in Appendix ii.

Of all growth media tested, the peat/vermiculite medium was decidedly superior. Comparisons of shoot and root weights in the peat/vermiculite medium reared with the three different nitrogen levels are presented in Tables I and II. At the bottom of each table, comparisons of Full N seedlings grown in the different soil media are included to illustrate the superior growth of seedlings in the peat/vermiculite medium. Error mean squares have been included in tables as an estimate of the variability associated with means. In these tables, and throughout the results section, an asterisk in the significance column denotes significance at the .05 level while NS indicates that differences were not significant at the .05 level.

Table I. Shoot growth in a P/V medium with three different nitrogen treatments and in three different rooting media with a Full N treatment.

Nitrogen treatment means (in a P/V medium)

	shoot weight (g)
N-	.034
N+	.132
Full N	.790
error mean square	.009

Orthogonal Comparisons:

	sig (.05)
Full N vs. N+ and N-	*
N+ vs. N-	*

Media treatment means (in Full N)

	shoot weight (g)
Peat/Vermiculite	.790
Vermiculite	.313
Peat/Sand/Vermiculite	.311
error mean square	.009

Orthogonal Comparisons:

	sig (.05)
P/V vs. V and S	*
V vs. S	NS

Table II. Root growth in a P/V medium with three different nitrogen treatments and in three different rooting media with a Full N treatment.

Nitrogen treatment means (in a P/V medium)

	root weight (g)
N-	.020
N+	.050
Full Nitrogen	.186
error mean square	.001

Orthogonal Comparisons:

	sig (.05)
Full N vs. N+ and N-	*
N+ vs. N-	*

Media treatment means (in Full N)

	root weight (g)
Peat/Vermiculite	.186
Vermiculite	.071
Peat/Sand/Vermiculite	.082
error mean square	.001

Orthogonal Comparisons:

	sig (.05)
P/V vs. V and S	*
V vs. S	NS

The three different nitrogen treatments produced clear separations among seedlings with respect to shoot and root growth. Table III illustratrates the effect of different nitrogen treatments on shoot/root ratios in the peat/vermiculite soil medium. No statistical analysis was performed.

Table III. Shoot/root ratios in a P/V medium with three different nitrogen treatments.

Nitrogen level	ratio means
N-	2.67
N+	2.80
Full N	4.30

After three months with Full N, roots were becoming crowded within planting containers, and the duration of future experiments was set at three to four months where similar growing conditions and container sizes were to be used.

As a base for future comparisons, nitrogen concentrations in leaves, roots, and stems were measured for plants reared in each nitrogen treatment. An ANOVA showed that nitrogen concentration was significantly related to nitrogen application (N-, N+, or Full N) and to plant part. Means for nitrogen concentrations are presented at the top of Table IV. The error mean square from the ANOVA has been included in the table as an estimate of the variability associated with nitrogen concentration means. Nitrogen

contents were extrapolated from nitrogen concentration means and plant weight means from Tables I and II (leaf weight was computed as 66% of shoot weight and stem weight was taken as the remainder of shoot weight). These means are presented at the bottom of Table IV.

Table IV. Nitrogen concentrations and contents of various plant tissues from seedlings reared with three different levels of nitrogen.

Nitrogen concentration means (% nitrogen)

(ERROR MEAN SQUARE .061)

NITROGEN LEVEL	PLANT PART leaf	root	stem
N-	1.94	1.83	1.34
N+	1.84	1.84	1.50
Full N	2.84	2.12	1.64

Total nitrogen means (mg of N)

NITROGEN LEVEL	PLANT PART leaf	root	stem	total
N-	.4	.4	.2	1.0
N+	1.6	.9	.7	3.2
Full N	14.8	3.9	4.3	23.0

No plants nodulated in response to the crushed-nodule inoculum.

PEAT EXPERIMENT

Nodulation

No Nodules developed on seedlings reared in commercial peats. Inoculum in the native peat medium did produce nodules but nodules occurred infrequently (see Table V).

Table V. The occurrence of nodules in native peat in three nitrogen treatments (no external source of inoculum applied).

ANOVA

SOURCE	df	MS	F	sig
Nitrogen	2	.135	1.93	NS
error	21	.070		

	Mean Nodule Occurrence
N-	25%
N+	19%
Full N	0%

The characteristics of nodules developed in the native peat medium (number, biomass, and fixation) are presented in Table VI.

Table VI. Characteristics of nodules developed on seedlings reared in native peat.

	nod #		nod biomass (mg)		fix/hr nm ethylene	
	mean	s.d.	mean	s.d	mean	s.d.
N-	1	0	6	0	.5	1
N+	2	2	10	8	4	6

Mean shoot and root weights of nodulated seedlings were larger than non-nodulated seedlings especially in the N+ nitrogen treatment (see Table VII).

Table VII. Shoot and root weights of nodulated and non-nodulated seedlings reared in native peat under two nitrogen treatments.

Shoot weight (g):

	N-		N+	
	mean	s.d.	mean	s.d.
Nod -	.06	.02	.15	.05
Nod +	.09	.04	.31	.03

Root weight (g):

	N-		N+	
	mean	s.d.	mean	s.d.
Nod -	.04	.01	.06	.02
Nod +	.06	.05	.10	.02

Growth in Different Peats

Rearing seedlings in different peats provided an opportunity to observe differences in seedling growth in various peat media. Growth means for seedlings reared in the different peat media are presented in Table VIII (only means for seedlings reared with Full N were calculated). ANOVAS for the whole experiment are included in Appendix iii, Table I).

Table VIII. Shoot weights, root weights, and shoot/root ratios of seedlings reared in five different peat media with a Full N treatment.

Means and Tukey HSD

Peat source	Shoots(g)		Roots(g)		Shoot/Root
Native	.934	b	.261	bc	3.63
Alberta Rose	1.277	a	.660	a	2.02
Sunshine (Old)	.892	b	.384	b	2.40
Sunshine (new)	.508	c	.175	c	2.93
Parkland	.871	b	.263	bc	3.46

The growth of seedlings in the Alberta Rose Peat was superior to seedling growth in other peats tested: shoot and root weights were greatest and shoot/root ratios were lowest.

B. ARTIFICIAL INOCULUM EXPERIMENT

GROWTH ROOM

Nodule Occurrence

An ANOVA (included in Appendix iii, Table II) was run to find how nitrogen and inoculum treatments affected the occurrence of nodulated seedlings. Nodule occurrence means are presented in Table IX. Although there were differences in nodulation with respect to nitrogen treatments, only inoculum treatments were statistically significant.

Table IX. The occurrence of nodulated seedlings in different nitrogen and artificial inoculation treatment combinations.

	proportion of nodulated seedlings		
Nitrogen Treatment	I -	I(1)	I(2)
N-	0%	65%	41%
N+	12%	76%	47%
Full N	6%	44%	38%

Fewer seedlings inoculated at 9 weeks (I 2) produced nodules than seedlings inoculated at 5 weeks (I 1) and nodulation was less than 100% with either inoculation. The nodules of uninoculated seedlings were not considered to be true nodules as none were able to reduce acetylene when tested.

Nodule occurrence was variable among experimental blocks and the variability appeared to correspond to a temperature difference within the growth room. This temperature difference was the result of the cooling system which forced cold air up at one end of the bench. Although temperatures were no more than 4° C different, the temperature difference reflected a difference in air turbulence within the growth room. At the cooler bench end air was circulating rapidly and transpirational stresses may have been placed on plants. It was reasoned that if growth room differences were great enough to affect nodulation, they should also have affected plant growth. Means for seedling shoot and root growth at the two bench ends were calculated. Root means did not differ with respect to bench ends but shoot means in the Full N treatment did show some differences although means were not significantly different at the .05 level (they were significant at the .13 level). Means for shoot growth and nodule occurrence with respect to bench position are presented in Table X.

Table X. Differences in shoot weights and nodule occurrence with respect to bench position in the Artificial Inoculum Experiment.

Shoot weight means (g)
(non-nodulated seedlings only)

Bench Position	N-	N+	Full N
warm	.02	.08	.97
cold	.02	.08	.76

Proportion of inoculated seedlings with nodules:

Bench Position	mean	s.d.
warm	69%	7%
cold	11%	10%

The Full N shoot weight mean for the cold bench position was about 20 percent less than the mean for the warm position and differences in nodule occurrence were pronounced.

Nodule Number and Biomass

ANOVAS were performed on nodulated plants to determine the effect inoculum and nitrogen treatments had on nodule characteristics. For nodule number, only inoculum treatments were significant, but nodule biomass responded significantly to both nitrogen and inoculum treatments. Means, calculated on a per plant basis, are presented in Table XI.

Table XI. The number and biomass of nodules per seedling in different nitrogen and inoculation treatments.

	MEANS	
NITROGEN	number	biomass(mg)
N-	5	3.2
N+	5	6.3
Full N	4	9.6
INOCULUM		
I (1)	2	9.5
I (2)	7	3.2

(ANOVAS are included in Appendix iii, Table III)

More nodules were produced with inoculation at 9 weeks (I 2) than at 5 weeks (I 1) although fewer seedlings became nodulated with the I (2) inoculation (see Table IX). Nodule biomass was greatest for seedlings reared with Full N and seedlings inoculated at I (1).

Fixation and Nodule Efficiency

Hourly fixation estimates had to be computed from measures of fixation made at a time interval that would provide an optimal estimate of fixation. No significant difference among estimates computed over a 10 hr time interval was found and, for reasons of convenience, a 2 hr time interval was selected as the basis for all hourly fixation estimates.

Fixation estimates for nodulated plants were statistically analyzed to observe the effects that nitrogen and inoculum treatments had on fixation. Means are presented in Table XII.

Table XII. Relative fixation rates of seedlings in different nitrogen and inoculation treatments.

	MEANS ethylene (nm) per hour
NITROGEN (NS)	
N-	0.6
N+	98.0
Full N	3.0
INOCULUM (NS)	
I (1)	67.0
I (2)	0.5

(The ANOVA is included in Appendix iii, Table IV)

Neither nitrogen nor inoculum treatments were statistically significant with respect to hourly fixation despite the large differences in means probably because error mean squares were extremely high. The standard error (square root of the error mean square) was 173% of the largest fixation mean.

Nodule efficiencies were calculated from hourly fixation rates and nodule biomass means. Nodule efficiency was calculated as micromoles of ethylene produced per gram of nodule (fresh weight). Nodule dry weights were converted to fresh weights with the conversion factor 6.5 used by Perry *et al.* (1979). Means are presented in Table XIII.

Table XIII. Relative nodule efficiences of seedlings in different nitrogen and inoculation treatment combinations.

	MEANS (micromoles of ethylene/hr/g nod fresh wt.)	
	I (1)	I (2)
N-	.03	.02
N+	3.20	.04
Full N	.05	.01

Table XIV presents nodule efficiency estimates for other non-leguminous nitrogen-fixing species.

Table XIV. Nodule efficiencies presented in the literature for various non-legume nitrogen-fixers (micromoles of ethylene/ hr/g nod fresh wt.).

SPECIES	FIXATION	AUTHOR
Shepherdia canadensis	2.9	(van Straten *et al.* 1977)
Alnus glutinosa	5-9	(Perry *et al.* 1979)
Alnus glutinosa	.2	"
Alnus rubra	26	(Carpenter *et al* 1979)
Alnus sinuata	12	"
Ceanothus velutinus	2.6	(McNab *et al.* 1979)

Table XV presents a table of correlations among fixation characteristics and nodule biomass and size (weight per individual nodule) characteristics (repeat observations were not pooled for this analysis).

Table XV. Correlations between hourly fixation and nodule biomass and between hourly fixation and nodule size.

	r values
Nod character	Fix/Hr
Nod biomass	.57
Nod size	.44

Fixation was significantly correlated with nodule biomass and nodule size, but correlations were not high.

Plant Growth

Analyses were run on plant characteristics to ascertain whether nitrogen and inoculum treatments (as they relate to nodulation) had an effect on plant growth. Inoculum and nitrogen treatments both significantly affected shoot weights, but root weights were only significantly responsive to nitrogen treatments (see Table XVI). No statistical analysis was performed on shoot/root ratios.

Table XVI. Pre-outplanting shoot weights, root weights, and shoot/root ratios of seedlings reared in different nitrogen and inoculation treatments.

Shoot wt. means (g)

MEANS AND TUKEY HSD (.05)

NITROGEN:

N-	.05	a
N+	.14	a
Full N	.98	b

INOCULUM:

I-	.37	a
I (1)	.49	b
I (2)	.32	a

	I-	I(1)	I(2)
N-	.02	.10	.02
N+	.09	.23	.10
Full N	.99	1.14	.82

(The ANOVA is included in Appendix iii, Table V)

Table XVI. Cont'd

Root wt. means (g)

MEANS AND TUKEY HSD (.05)

NITROGEN:		
N-	.02	a
N+	.05	a
Full N	.32	b

INOCULUM:	
I-	.14
I(1)	.14
I(2)	.11

	I-	I(1)	I(2)
N-	.02	.03	.01
N+	.05	.06	.05
Full N	.35	.34	.28

(The ANOVA is included in Appendix iii, Table V)

Table XVI Cont'd

shoot/root ratio means

NITROGEN:	
N-	1.88
N+	2.41
Full N	3.16
INOCULUM:	
I -	1.99
I (1)	3.13
I (2)	2.31

Shoot and root weight means for seedlings inoculated at 9 weeks (I 2) were smaller than means of uninoculated (I -) seedlings in the Full N treatment. Full N seedlings were analyzed further to determine whether smaller plant size was related to nodulation (see Table XVI a).

Table XVI a. Pre-outplanting shoot and root weights of seedlings reared in different inoculation treatments with Full N.

	Mean shoot weight (g) nod -	nod +
I-	1.05	–
I(1)	1.27	1.27
I(2)	.79	1.12

	Mean root weight (g) nod-	nod +
I-	.34	–
I(1)	.31	.37
I(2)	.22	.36

It would appear that the low shoot and root weight means for Full N seedlings inoculated at I (2) were the result of low weights of non-nodulated seedlings.

Correlations between nodule biomass and shoot weight were calculated for nodulated seedlings in each nitrogen treatment to determine how closely shoot weight and nodule biomass were related. Correlations between nodule biomass

and shoot weight for seedlings in the N- and N+ treatments were very high (r^2 greater than .90), while shoot weights of seedlings provided with Full N were relatively unrelated to nodule biomass (r^2 less than .30). Root weights were most related to nodule weights when roots were supplied with no nitrogen (r^2 equals .93) and with rising nitrogen levels the relationship decreased to an r^2 value of .57 and .28 for N+ and Full N treatments respectively. Observations within blocks were not pooled for this analysis. (Complete regression statistics are presented in Appendix iii, Table VI).

ANOVAS were run on several nitrogen measures: %N in oldest living leaves; %N in top leaves; %N in roots; %N in stems; total N in leaves; total N in roots; total N in stems; and total N in whole plants to find how these measures reflected nitrogen status. The nitrogen measures most responsive to both nodulation and nitrogen treatments were total nitrogen in roots, stems, and whole plants. The means and standard deviations for some of the nitrogen measures are listed in Table XVII.

Table XVII. Pre-outplanting nitrogen contents of nodulated and non-nodulated seedlings reared in three different nitrogen treatments.

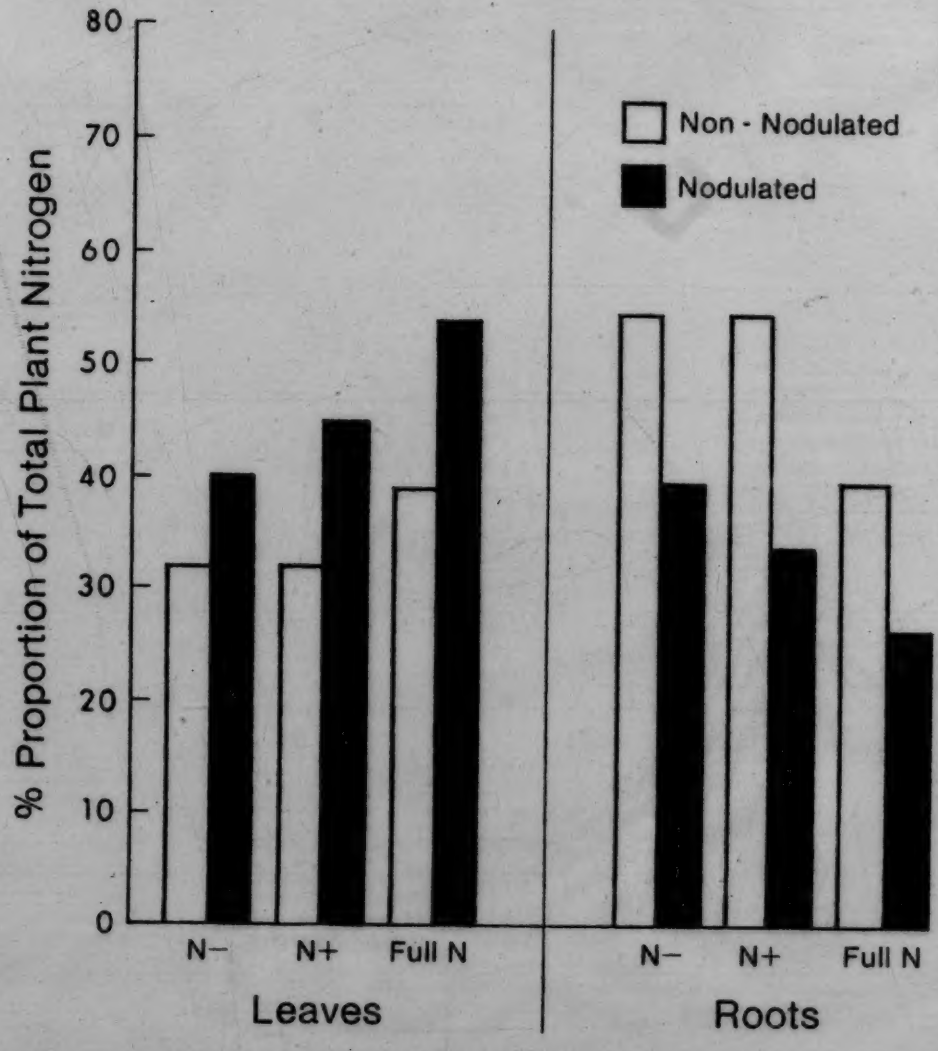
		Total N (mg/seedling)					
		N-		N+		Full N	
		Mean	s.d.	Mean	s.d.	Mean	s.d.
leaf	nod(-)	.1	.03	.4	.1	5.5	3.9
	nod(+)	.3	.2	1.1	.7	11.9	3.6
root	nod(-)	.2	.06	.7	.3	4.5	1.4
	nod(+)	.2	.03	1.0	.2	5.6	.5
stem	nod(-)	.06	.01	.2	.06	2.4	1.2
	nod(+)	.1	.09	.7	.1	3.9	.3
whole plant	nod(-)	.4	.1	1.3	.5	12.2	7.1
	nod(+)	.6	.3	3.3	.7	21.4	4.5

As supplied nitrogen increased (N-, to N+, to Full N), total nitrogen content increased and a similar trend was evident between nodulated and non-nodulated plants within each nitrogen treatment— nodulated plants contained more nitrogen than non-nodulated plants. The nitrogen concentration of seedlings was not as responsive to increased nitrogen as nitrogen content because increased nitrogen was associated with increased plant growth 'diluting' nitrogen concentrations within tissues.

The distribution of nitrogen within plants was calculated from nitrogen content means to examine how nitrogen distributions were affected by available nitrogen

supplies. As nitrogen supplied to plants was increased either through nitrogen provided exogenously or nitrogen fixed, relatively more went into leaves than into roots (see Figure 1). This difference corresponds to the increasing shoot/root ratios observed as seedlings were provided with more nitrogen (see Table XVI).

Figure 1. The Distribution of Nitrogen Within Seedlings.



OUTPLANTING

Outplanting Treatments

There were no significant differences among outplanting treatments with respect to survival, growth, or nodulation after outplanting.

Nodule Occurrence

After outplanting, virtually all plants became nodulated with the only exceptions being several plants in the N- treatment.

Nodule Number and Biomass

Table XVIII presents nodule number and biomass means for all nodules observed after outplanting (this includes nodules that developed over the entire growth room/outplanting period and nodules that developed only after outplanting). For the nodulation characteristic 'number of nodules per plant', only nitrogen treatments produced significant differences among seedlings but both inoculum and nitrogen treatments significantly affected nodule biomass.

Table XVIII. The number and biomass of nodules per seedling after outplanting on seedlings reared in different growth room nitrogen and inoculation treatments.

Mean nodule number per plant
(with Tukey HSD)

NITROGEN TREATMENTS:	number of nodules	
N-	5	a
N+	14	a
Full N	48	b

INOCULUM TREATMENTS	
I -	9
I (1)	23
I (2)	35

	I-	I(1)	I(2)
N-	1	6	8
N+	5	14	22
Full N	22	49	74

(The ANOVA is included in Appendix iii, Table VII)

Table XVIII. Cont'd

Mean nodule biomass per plant
(with Tukey HSD)

NITROGEN TREATMENTS:	nod biomass(mg)	
N-	5	a
N+	20	b
Full N	54	c

INOCULUM TREATMENTS:		
I -	8	a
I (1)	38	b
I (2)	32	b

	I-	I(1)	I(2)
N-	2	13	1
N+	4	35	19
Full N	20	67	75

(The ANOVA is included in Appendix iii, Table VII)

The Full N/(I 2) treatment combination was associated with the production of the greatest number of nodules and the greatest nodule biomass.

Table XIX presents estimates of the increase in nodulation after outplanting for seedlings reared with Full N only.

Table XIX. Increases in nodule number and biomass per seedling after outplanting for seedlings reared with Full N during the growth room period.

	MEAN NET INCREASES	
	nod number per plant	nod biomass (mg) per plant
I -	22	20
I (1)	45	51
I (2)	66	66

Seedlings inoculated at I (2) produced greater net increases in nodule number and nodule biomass after outplanting than seedlings inoculated at I (1) and both I (1) and I (2) treatments produced more nodules and greater nodule biomass than uninoculated (I-) seedlings.

Plant Growth

Table XX presents means and Tukey HSD values for nitrogen and inoculum treatment effects on root and shoot growth over the entire growth room/outplanting period (approximately six months). Shoot/root ratios were not significantly different among seedlings in different nitrogen or inoculum treatments.

Table XX. Shoot and root weights after outplanting for seedlings reared in different nitrogen and inoculation treatments.

MEANS AND TUKEY HSD

SHOOT WEIGHT (g)

NITROGEN TREATMENTS:		
N-	.13	a
N+	.36	b
Full N	1.09	c

INOCULUM TREATMENTS:		
I -	.40	b
I (1)	.63	a
I (2)	.54	b

(means for watered and clipped outplanting treatments only)

	I-	I(1)	I(2)
N-	.04	.25	.08
N+	.20	.50	.46
Full N	1.19	1.31	1.26

(The ANOVA is included in Appendix iii, Table VIII)

Table XX. Cont'd

MEANS AND TUKEY HSD

ROOT WEIGHT (g)

NITROGEN TREATMENTS:		
N-	.08	a
N+	.26	b
Full N	.83	c
INOCULUM TREATMENTS:		
I -	.30	a
I (1)	.46	b
I (2)	.42	ab

	I-	I(1)	I(2)
N-	.02	.17	.06
N+	.13	.39	.27
Full N	.76	.82	.93

(The ANOVA is included in Appendix iii, Table VIII)

Table XXI presents estimates of net shoot and root weight increases after outplanting.

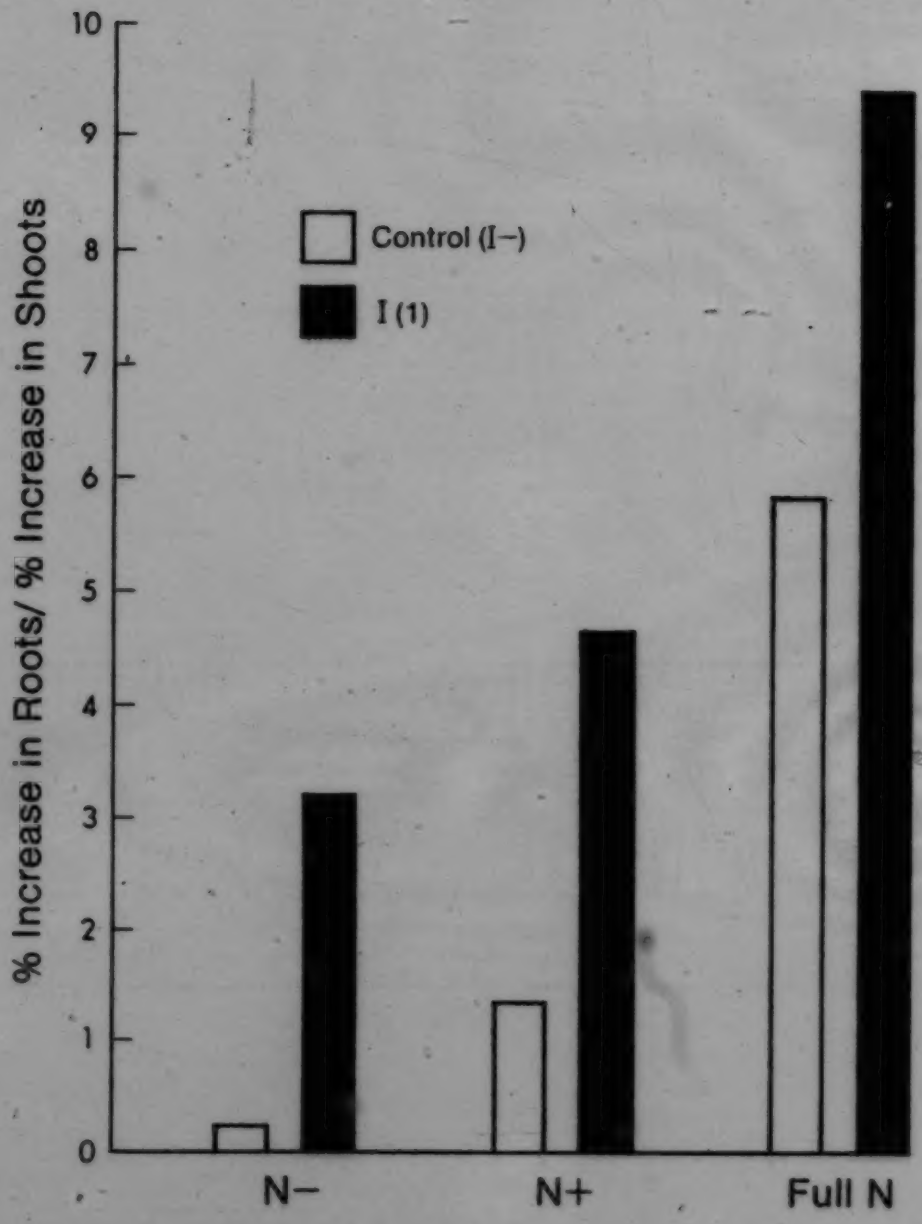
Table XXI. Increases in shoot and root weights after outplanting for seedlings reared in different nitrogen and inoculation treatment combinations.

	MEANS (g)		
	I -	I (1)	I (2)
shoot wt.			
N-	.03	.16	.06
N +	.11	.27	.36
Full N	.20	.17	.44
root wt.			
N-	+	.14	.05
N +	.08	.33	.22
Full N	.41	.48	.65

For Full N treatments, roots grew considerably more than shoots after outplanting and I (2) seedlings had greater increases in both shoots and roots than I - or I (1) seedlings.

A general trend observed was that seedlings with access to more nitrogen before outplanting, either through nitrogen provided in fertilizer solutions or through nitrogen fixed, produced relatively more root growth than shoot growth after outplanting (see Figure 2).

Figure 2. Relative Growth of Roots and Shoots After Outplanting.



Survival

Table XXII presents means for percent survival as it related to nitrogen and inoculum treatments. This table is based only on survival estimates of the nine blocks removed for the Outplanting analysis. Survival estimates were based on greenhouse survival and survival after the first growing season combined.

Table XXII. The proportion of seedlings surviving the entire growth room and outplanting periods in different nitrogen and inoculation treatments (for the nine experimental blocks sampled).

MEANS AND TUKEY HSD (.05)

NITROGEN:	SURVIVAL	
N-	69%	a
N+	87%	b
N Full	87%	b

INOCULUM:	SURVIVAL	
I -	65%	a
I (1)	93%	c
I (2)	85%	b

(The ANOVA for the analysis is included in Appendix iii, Table X)

Means of percent mortality for all seedlings over the entire growth room and outplanting experiment are presented in Table XXIII.

Table XXIII. Mortality over the growth room and outplanting periods for seedlings reared in different nitrogen and inoculation treatment combinations (for all seedlings outplanted).

	Growth room	Outplanting
N-		
I -	15%	48%
I (1)	12%	13%
I (2)	8%	28%
N+		
I -	6%	11%
I (1)	6%	4%
I (2)	3%	0%
Full N		
I -	10%	3%
I (1)	9%	1%
I (2)	12%	0%

For Full N treatments, mortality in the growth room was greater than mortality in the field. Only in the N- and N+ treatments did field mortality appear to be reduced in conjunction with inoculum treatments.

V. DISCUSSION: STUDY I

A. INITIAL GROWTH AND NODULATION EXPERIMENTS

The purpose of the Initial Growth and Nodulation Experiments was to provide basic information to assist with the design of the Artificial Inoculum Experiment. The nitrogen treatments in the Preliminary Growth Experiment were designed to induce different degrees of nitrogen deficiency in seedlings. These treatments produced distinct separations among seedlings with respect to growth and nitrogen uptake (see Tables I and II) and the treatments were retained unadjusted for use in the Artificial Inoculum Experiment. Seedlings were able to grow in peat media without any supplied nitrogen and the Fertilizer Experiment demonstrated that the Full N treatment was not damaging to seedlings. Different rooting media were tested to see which media produced the best seedling growth in the greenhouse. A peat/vermiculite medium was superior to a vermiculite medium for rearing *Shepherdia* so only a peat/vermiculite medium was used for the rearing of seedlings in the Artificial Inoculum Experiment. The difference in growth between a vermiculite and a peat/vermiculite medium may be an important finding for other researchers who have been using vermiculite or similar media for rearing seedlings to be used in inoculum tests. There is a possibility that seedlings reared in these media will not represent vigorous seedlings. Seedling growth in Alberta Rose Peat, the peat used for rearing *Shepherdia*

in the Artificial Inoculm Experiment, was compared to seedling growth in other peats. The Alberta Rose appeared to be a satisfactory commercial peat for rearing *Shepherdia* as evidenced by the superior growth of seedlings in that peat (see Table VIII).

Seedlings reared in the Preliminary Growth Experiment were given meticulous care. Growth was carefully monitored to ensure that seedlings would be healthy and vigorous, and seedlings reared in the Preliminary Growth Experiment compared favourably to *Shepherdia* seedlings observed in other greenhouses. Comparisons between seedlings reared in the Preliminary Growth Experiment and seedlings reared in the Artificial Inoculum Experiment provided satisfaction that the seedlings used to test the artificial inoculum were healthy and vigorous. Seedlings reared in the Artificial Inoculum Experiment were reared under a growing regime that had been tested and refined in the Preliminary Growth Experiment and although seedling growth in the two experiments was very similar, some differences were apparent. Seedlings in the Full N treatment of the Artificial Inoculum Experiment were larger than seedlings in the Full N, peat/vermiculite treatment of the Preliminary Growth Experiment. This may have been due to the higher light intensities used in the Artificial Inoculum Experiment. Plants were also slightly older in the Artificial Inoculum Experiment. Nitrogen concentrations and contents of seedling tissues were higher in the Preliminary

Growth Experiment where total amounts of fertilizer were greater and fertilizer applications were more frequent (this may have reduced leaching losses). Plants in the Artificial Inoculum Experiment were becoming chlorotic near the end of the growth room rearing period probably because of the infrequent nitrogen applications that seedlings recieved over that time (nitrogen applications were suspended ten days before and after inoculation and two weeks before acetylene reduction tests were made), and it is important to note that by the time seedlings were outplanted, they did not contain maximum levels of nitrogen within their tissues even in the Full N treatment.

Evidence provided by the Initial Growth and Nodulation Experiments strongly suggested that nodules do not develop readily on greenhouse-reared *Shepherdia* seedlings without a supplied inoculum. In the Preliminary Growth Experiment, no nodules developed on seedlings even when a crushed nodule inoculum was applied. Air-drying nodules may have killed nodule endophytes, or phenolics or other inhibitory substances may have been released from nodules upon crushing (Baker *et al.* 1979; Baker and Torrey 1979). Phasic variations in nodule endophytes, if these occur, may hinder nodulation at particular collection times (Gardner and Bond 1957) and this may have contributed to unsuccessful nodulation. Apparently, other workers have also had difficulty infecting plants with crushed nodules in peat media (M. Lalonde pers. comm.). No nodulation was observed

in any commercial peats tested. Either the source of inoculum was absent from peat deposits at mining, or processing of the peat was too severe to maintain viable endophytes. Nodulation did occur in native peat but the frequency of nodule occurrence was low. This may have been due to the quality or abundance of inoculum contained in the native peat, or it may have been due to growth room conditions (seedlings in the Peat Inoculum Experiment were reared in the 'cold' section of the growth room bench). The few observations of nodules obtained in native peat precluded comparisons with nodules induced by the artificial inoculum.

B. ARTIFICIAL INOCULUM EXPERIMENT

NODULE OCCURRENCE

Growth Room

Although nodules did develop on inoculated seedlings over the growth room period, not all inoculated seedlings nodulated. Gardner and Bond (1957) used a crushed nodule inoculum to inoculate *Shepherdia* and their inoculation rate was virtually 100%, and researchers using crushed-nodule inocula for other species have reported similar success (Bond and MacKintosh 1975; Rodriguez-Barrueco *et al.* 1970; Stewart and Bond 1961). In all of the above cases, crushed nodule inocula were applied directly to seedling roots so that root/inoculum contact was assured. Lalonde and Calvert (1979) achieved 100% nodulation in inoculated *Alnus* seedlings using an artificial inoculum but these nodulation tests were performed in hydroponics or in an arcillite potting medium and not a peat medium.

Several factors may have been responsible for less than complete nodulation using the artificial inoculum, but without further testing it is not possible to say which factors may have been most important:

1. The inoculum used was from *Eleagnus umbellata* not *Shepherdia* and host/endophyte incompatability may have been responsible for low nodulation (Becking 1977). Dawson and Gordon (1979) speculated on this in their experiments with two strains of *Alnus*

glutinosa.

2. Less than ideal growing conditions in the growth room could have contributed to overall poor nodulation in the experiment considering that 69% of inoculated seedlings produced nodules in the warm growth room section as opposed to only 11% in the cold section (see Table X).
3. The peat medium may have acted as a barrier between endophytes and roots, or the high cation exchange capacity of the peat may have immobilized endophytes (Alexander 1977). The containers used for rearing seedlings were well-drained and seedlings were watered frequently with large amounts of water and leaching may have removed considerable inoculum several days after application. If endophyte/root contact was a major cause of poor nodulation, the technique for administering the inoculum should be changed. This could be accomplished by increasing the total inoculum dosage, admininistering inoculum over several applications, or by changing the application method so that roots are put in direct contact with inoculum.

Fewer seedlings produced nodules in response to inoculation at 9 weeks (I 2) than inoculation at 5 weeks (I 1) (see Table IX). In order to schedule future inoculations properly, it is important to assess whether this observation was due to an inherent developmental

change in seedlings or to changes peculiar to conditions in the Artificial Inoculum Experiment. Again, it is not possible to determine the exact reason for the difference in nodulation but several suggestions can be examined:

1. The inoculum used at each inoculation was from a different preparation and, although each inoculum was prepared in the same manner, some differences may have existed. There were also different lag times between the preparation and injection of inocula (the second inoculum was stored longer before use). It was unfortunate that the experiment had to be designed so that staggered inoculations were required, but this was unavoidable because seedlings had to be outplanted at the same time and developmental stage. Future experiments could be designed so that seedling development is staggered and inoculum preparations are applied at the same time however, unless growth conditions are rigidly controlled, seedling differences rather than inoculum differences will confound the interpretation of results—the problem will not be easily resolved.
2. Plant vigour may be an important factor for plant nodulation. This was suggested by corresponding differences in plant growth and nodule occurrence with bench position (see Table X). At the first

inoculation, most seedlings would have had similar vigour but at the second inoculation plants would be growing more rapidly with vigour differences more apparent. Vigour may have even been reduced at the second inoculation because seedlings had been through an extended period without fertilizer (fertilizer applications were suspended 10 days before and after each inoculation). The hypothesis that lower nodulation at the second inoculation was due to lower plant vigour is supported by observations in the three nitrogen treatments. The drop in nodule occurrence was most pronounced in N- and N+ treatments where plant vigour would have been considerably reduced by an additional month of nitrogen deprivation, while the difference between first and second inoculations was not so marked in the Full N treatment. However, the vigour hypothesis is weakened by the fact that after outplanting virtually all seedlings nodulated.

3. Developmental differences in the susceptibility of *Shepherdia* and *Hippophae* (both members of the Elaeagnaceae) to nodulation at different developmental stages have been reported (Bond and MacConnell 1956; Gardner and Bond 1957; Gardner 1958). However, in these cases nodulation was reduced at earlier stages and enhanced at later developmental stages. Developmental differences of

seedlings in the Artificial Inoculum Experiment do not adequately explain nodulation differences for two reasons. First, although fewer seedlings became nodulated in the I (2) treatment, those seedlings that did nodulate produced more nodules than seedlings inoculated in the I (1) treatment and second, almost all seedlings nodulated after outplanting. It is possible that some nodules initiated after the second inoculation simply went unnoticed because of their incomplete development.

There were no significant differences among nitrogen treatments with respect to the occurrence of nodulated seedlings although nodule occurrence was somewhat less than the overall average in the Full N treatment. Generally, it has been observed that some exogenous nitrogen enhances nodule development while large amounts (close to the amounts required by seedlings for full growth) reduce or inhibit nodule development (Bond *et al.* 1954; Rodriguez-Barrueco *et al.* 1970; Stewart and Bond 1961). This effect varies among species and it is also dependent on the nature of the medium in which plants are grown (Virtanen and Miettinen 1963). Nodulation in *Hippophae* and *Shepherdia* seedlings, appears to be very sensitive to exogenous nitrogen relative to *Alnus* or *Myrica* seedlings (Bond *et al.* 1954; Bond and MacConnell 1956; Gardner and Bond 1957; Gardner

1958). A possible reason for the occurrence of nodulation in the Full N treatment of the Artificial Inoculum Experiment is that the effective supply of nitrogen to seedlings was not high enough to inhibit nodulation. The peat medium would have been able to 'hold' applied ammonium-nitrogen through its high cation exchange capacity (Knowles 1977) and plants would not have been able to readily absorb nitrogen as is the case in hydroponic cultures where ions simply diffuse into roots (Black 1968). Much of the nitrate-nitrogen applied would likely have been leached soon after application as a result of large water applications and rapid container drainage. Although 35 mg of nitrogen were applied to each Full N seedling, perhaps much smaller amounts were actually accessible to roots. Seedlings were also growing rapidly in the Artificial Inoculum Experiment and, as was previously discussed, nitrogen levels within plant tissues were less than maximum. If carbon/nitrogen ratios within plant tissues are critical to nodule occurrence, the high growth and subsequent low nitrogen concentration of seedlings in the Artificial Inoculum Experiment may have allowed nodulation to occur (Gardner and Bond 1957). The finding that *Shepherdia* can nodulate when nitrogen is provided in quantities adequate for large seedlings to be produced is very significant because smaller seedlings, even though they may be nodulated and eventually able to produce substantial

growth, would likely have difficulty competing with other vegetation on reclamation sites.

The occurrence of nodule-like structures was an interesting find, but because no anatomical analyses were performed on these nodules nothing can be inferred about them (*i.e.* whether they were the result of ineffective nodulation, whether they were just hormonal anomalies (Bermudez de castro *et al.* 1977), or whether they were the result of some other influence).

Outplanting

After outplanting for 2.5 months, virtually all plants sampled were nodulated. The only plants that did not produce nodules were uninoculated N- seedlings which were very small and chlorotic at outplanting. Peat, overburden, or native sand at the outplanting site must have contributed the inoculum. Field observations on other species indicate that although nodulation may not be 100% immediately after a fixer is established, it can reach 100% over time (Youngberg *et al.* 1979). Thus, even in outplanting areas where native inoculum is not abundant, nodulation can likely progress over time. Although 100% nodulation appears to be possible without any pre-outplanting inoculation, some inoculum may be desirable due to the quality of native inoculum or due to the effects of nodulation on plant growth at outplanting. Plants nodulated at outplanting could

contribute to soil inoculum supplies through endophyte liberation at nodule senescence, but extra-nodular growth is slow and this contribution may not be great (van Dijk 1979).

NODULE NUMBER AND BIOMASS

Growth Room

At the end of the growth room rearing period, seedlings inoculated at I (2) produced more nodules than seedlings in the I (1) treatment (see Table XI) even though fewer seedlings became nodulated in the I (2) treatment (see Table IX). Reasons for this apparent contradiction are not clear. Differences could be due to either host plant or inoculum factors but, regardless of the cause, more infections took place on seedlings inoculated at 9 weeks than at 5 weeks. This could have occurred for several reasons:

1. Because roots were larger at the second inoculation there may have been more infection points for nodules to develop at.
2. Nitrogen requirements may have been greater at the second inoculum and plants may have been more susceptible to multiple infections.
3. Plants may not have inhibited infections after initial contact due to increased nitrogen demand or some other developmental reason.
4. Plants may have had more photosynthate available for nodule development at the second inoculum because of

increased leaf area and rapid growth.

The nodule biomass developed from the first inoculation was larger than the biomass developed after the second inoculation probably because nodules had been induced one month earlier. Seedlings in the Full N treatment produced greater nodule biomass than seedlings reared with less nitrogen. As was previously discussed, development of nodules supplied with nitrogen may be greater than nodules supplied with no nitrogen at least in some species (Bond *et al.* 1954; Rodriguez-Barrueco *et al.* 1970; Stewart and Bond 1961). Full N plants were more vigorous than N- or N+ plants and they would likely have produced more photosynthate for use in nodule development. Dawson and Gordon (1979) found high correlations between photosynthesis and nodule biomass. Gardner and Bond (1957) commented that the *Shepherdia* seedlings in their experiment were not growing in ideal conditions and perhaps their finding that *Shepherdia* nodulation was very sensitive to nitrogen was only true for the growing conditions of their experiment.

Outplanting

After outplanting, seedlings provided with Full nitrogen and the second inoculum produced the greatest nodule numbers and biomass (see Table XVIII). Nodule biomass increased over 300% for I (1)seedlings and over 1000% for I (2) seedlings in the Full N treatment (see

Table XIX). The high rates of increase after outplanting probably reflected the greater allocation of photosynthate to roots, evidenced by a decrease in shoot/root ratios, after outplanting (Dawson and Gordon 1979; Gordon and Wheeler 1978). Larger growth increases for nodules induced at I (2) may only have reflected that there were more nodules developing on plants inoculated at I (2) and perhaps the younger I (2) nodules were growing more rapidly than older I (1) nodules.

NODULE EFFICIENCY

Symbiotic nitrogen-fixing organisms have regulatory mechanisms that enable them to cease fixation and use exogenous nitrogen sources when available (Brock 1979). Fixation per plant was greatest for inoculated plants supplied with small amounts of nitrogen (*i.e.* seedlings in the N+ treatment) (see Table XIII). These plants would have been stimulated to fix nitrogen as a result of low available nitrogen supplies but they weren't so small and chlorotic that fixation was impeded. Seedlings that nodulated in the N- treatment generally did not fix nitrogen at high rates and this was likely related to their non-vigorous state. Nodule efficiency decreased when nitrogen supplies approached levels adequate for full seedling growth which is a common observation (Rodriguez-Barrueco *et al.* 1970; Stewart and Bond 1961). The efficiency of nodules established after the second inoculation was probably lower

than nodules established after the first inoculation because of the shorter development period. The efficiency of *Shepherdia* nodules is comparable to the efficiency of other non-leguminous nitrogen-fixers (see Table XIV).

No significant differences among hourly fixation estimates computed over a ten hour time interval were found. Perhaps *Shepherdia* exhibits a long period of linearity after excision or, because nodules were attached to intact roots, photosynthate supplies may not have been limiting to fixation over a 10 hr period.

Correlations among physical nodule characteristics and fixation estimates were significant but not high (see Table XV). Nodule size (weight per individual nodule) and fixation rates were correlated, with larger nodules generally fixing more nitrogen. However, because nodules were only just developing, correlations between nodule size and fixation may only have reflected differences in the stage of nodule development. Also, as nodules develop over time, the microbe proportions within nodules would be expected to change and reduce this correlation (Sprent 1979).

PLANT RESPONSE

Growth Room

The results showed clearly that as levels of supplied nitrogen increased, plant weight increased. A similar trend was observed with inoculum treatments: inoculated seedlings were larger than uninoculated seedlings (although this was not pronounced in Full N treatments). After investigations of plant nitrogen, it was clear that inoculation (as it relates to nodulation) increased plant nitrogen and increases in plant growth could be attributed to nitrogen increases (see Table XVII). However, it is understood that nodulation may be associated with effects on plant growth other than nitrogen (Alexander 1977; Bermudez de Castro *et al.* 1977; Brock 1979; Pelczar *et al.* 1977).

Inoculation increased shoot and root weights of seedlings in all nitrogen treatments except for the Full N/ (I 2) treatment. Shoot and root weight means of Full N/ (I 2) seedlings were less than uninoculated (I-) seedling means but the lower means were due to small non-nodulated seedlings (means of nodulated seedlings in the I (2) treatment were larger than uninoculated seedling means) (see Tables XVI and XVI a). The observation that non-nodulated seedlings in the I(2) treatment were small was probably attributable to sampling error and not to the inoculation treatment.

Correlations between shoot weight and nodule

biomass for seedlings receiving the same N- or N+ treatment were high (r^2 greater than .90). Pinchbeck *et al.* (1980) found high correlations between nodulation and juvenile plant vigour demonstrating that larger plants produced more nodules. Dawson and Gordon (1979) showed that photosynthate production was highly correlated with nodule biomass. All of these authors inferred that plant biomass influenced nodule biomass. However, relationships between nodule biomass and shoot weight in the Artificial Inoculum Experiment were likely indicative of benefits to seedlings from nitrogen-fixation as well as benefits to nodules from carbon-fixation. Seedlings receiving a Full N treatment had poor correlations with nodule weight and, if nodule/plant biomass correlations reflected only the response of nodules to carbon and not plants to nitrogen, nodule biomass should have corresponded to shoot weight in all nitrogen treatments. Root and nodule biomass correlations were not as strong as shoot and nodule biomass correlations but, when nutrients and water are abundant, high nitrogen levels favour shoot growth over root growth (Black 1968). If nodule biomass only reflected the transfer of photosynthate to nodules, root weights should have been as good an indicator of photosynthate transfer to nodules as shoot weights.

Nitrogen did not accumulate in plant tissues— it was converted to plant biomass and, as plant nitrogen

increased through additions of fertilizer or through nitrogen-fixation, proportionally more nitrogen went into leaves than roots (see Figure 1). In the growth room then, increases in nitrogen were associated with larger increases in shoot weights than root weights. However, over this period of seedling development, other growth factors (water and other nutrients) were in abundance and shoot growth was unimpeded.

Outplanting

After outplanting, seedlings in the N- and N+ treatments inoculated at I (1) produced more root and shoot biomass than uninoculated seedlings or seedlings inoculated at I (2) in those nitrogen treatments. In the Full N treatment, seedlings inoculated at I (2) had shoot weights intermediate to uninoculated and I (1) seedlings (differences weren't statistically significant). Root weight means for Full N seedlings inoculated at I (2) were larger than uninoculated or I (1) means but again differences were not statistically significant. Absolute increases in plant weights over the outplanting period appeared to be greatest for Full N seedlings inoculated at I (2). However, if the sample of I (2) seedlings analyzed before outplanting was not representative of the entire experiment (as was previously suggested) these increases may be an over-estimate of growth increases in that treatment.

For all nitrogen treatments, seedlings that had

access to more nitrogen before outplanting produced greater relative gains in roots than shoots after outplanting. This was opposite to what was found after the growth room rearing period where increased nitrogen was associated with increased shoot growth. The divergent trends may have been due to some physiological 'outplanting' response or the difference may be explained by the carbohydrate balance theory described by Black (1968). Nitrogen was rapidly converted into growth products in the growth room and, because of rapid growth, little carbohydrate was stored. After outplanting water and nutrients became limiting and, because water was more limiting to shoots than roots (shoots experience a greater transpirational pull and they are further from the water source), shoot growth slowed. This would have been especially true for seedlings with large shoot/root ratios at outplanting (*i.e.* nitrogen rich seedlings). Shoot carbohydrate was then in excess of shoot needs, and more carbohydrate was transferred to roots for root growth. Regardless of the explanation, the point to be made is that nitrogen, whether obtained from the environment or through fixation, does not determine plant growth response it only enhances the response. There is no need for concern that seedlings fixing their own nitrogen will continue to increase shoot growth more than root growth once seedlings are outplanted.

It was really too early to assess seedling survival because seedlings had not had an over-wintering period. A preliminary assessment indicated that in N- and N+ treatments, inoculation aided field survival however, uninoculated seedlings, or seedlings that didn't nodulate in those treatments were very small and chlorotic. Full N seedlings experienced very little mortality after outplanting in all inoculation treatments (see Table XXI).

VI. STUDY II: INTRODUCTION

There is presently interest in the use of actinomycete-nodulated angiosperms for ameliorating nitrogen deficiencies in wildland soils, but this interest is limited by a lack of knowledge and experience in the management of these species (Debell 1979; Jurgensen *et al.* 1979; Mellilo and Aber 1979; Newton and Howard 1979; Rottink 1979). Many studies have investigated nitrogen accrual beneath actinomycete-nodulated angiosperms, and it does appear that these shrubs increase total soil nitrogen supplies (Bollen and Lu 1968; Daly 1966; Newton *et al.* 1968; Voight and Steucek 1969; Youngberg and Wollum 1976), however, organic nitrogen must be mineralized into simpler compounds, usually ammonium or nitrate, before it can be used by other plants (Black 1968). Evidence indicates that nitrogen-rich litter beneath actinomycete-nodulated plants does mineralize readily (Bollen and Lu 1968; Cromack *et al.* 1979), and several investigations have suggested that non-fixing plants adjacent to nitrogen-fixers do obtain nitrogen benefits (Tiffney and Barrera 1979; Youngberg *et al.* 1979; Zavitkovsky *et al.* 1979). In order to obtain nitrogen benefits, mixed plantations of nitrogen-fixing and non-fixing woody species must be established under the following considerations: 1) the fixing shrub must have a high nitrogen-fixing rate and 2) distances between fixers and non-fixers must be based on the horizontal distance that fixers are effectively able to supply nitrogen to non-fixers (Voight and Steucek 1969;

Zavitkovsky *et al.* 1979). Effective horizontal distance for nitrogen transfer will be dependent on root lengths of both fixers and non-fixers, and on wind dispersal of fixer litter (Zavitkovsky *et al.* 1979).

The following characteristics of *Shepherdia* will be important for determining its contributions to soil nitrogen:

1. *Shepherdia*'s fixation rate,
2. the distribution, composition, and amount of *Shepherdia* litter,
3. the concentration of nitrogen in *Shepherdia* litter and in organic and mineral soil layers beneath *Shepherdia*, and
4. the decomposition and subsequent mineralization of nitrogen from *Shepherdia* litter residues.

Shepherdia's fixation rate is comparable on a nodule weight basis to fixation rates of other nodulated non-legumes (see Tables XIII and XIV included in the Results section of Study I), so only the characteristics of *Shepherdia*'s nitrogen-containing residues need be considered with respect to soil nitrogen contributions.

This study was designed to satisfy the following objectives:

1. to document what *Shepherdia* litter is comprised of and how that litter is distributed,
2. to investigate nitrogen concentrations in the litter of

Shepherdia and other shrubs and to investigate nitrogen concentrations in soil residues beneath *Shepherdia* and other species, and

3. to investigate how readily *Shepherdia* residues decompose and mineralize nitrogen relative to other species.

The study will consider only the above-ground nitrogen contributions of *Shepherdia* because the quantification of root senescence and root exudates is extremely difficult, if not impossible, under natural field conditions.

VII. STUDY II: METHODS

The field study was conducted near Fort McMurray, Alberta so that results would be more relevent to reclamation activities in that area. The climate in the Fort McMurray region is much harsher than the climate of the Pacific Northwest region where the bulk of studies on non-legume nitrogen contributions have been carried out. This is important to note when examining the results obtained in this study relative to results obtained in most other studies of soil nitrogen effects. The Fort McMurray area has a short growing season and low precipitation:

-mean annual temperature 0° C

-2,154 growing season degree days

-frost free period from June 19 to Aug 19

-effective growing season from May 1 to Sept 1

-mean annual precipitation 45 cm with 30 cm falling over the growing season for a mean annual deficit of 4.3 cm (Longley 1972).

A study site was selected based on the following criteria:

1. typical of *Pinus/Populus/Shepherdia* sites
2. sandy soil
3. presence of both *Shepherdia* and *Alnus crispa* (Ait.) Pursh

The presence of both *Shepherdia* and *Alnus* was necessary in order to make comparisons between the two nitrogen-fixing shrubs. Soil comparisons were based on differences among

soils beneath nitrogen-fixing shrubs and beneath areas with only small ericaceous shrubs. Soil comparison groups are referred to as shrub types and they consist of the following divisions:

1. *Shepherdia* (for soils with a *Shepherdia* cover)
2. *Alnus* (for soils with an *Alnus crispa* cover)
3. Open (for soils with only an ericaceous shrub cover).

(Vegetation beneath nitrogen-fixing shrubs was very similar to the vegetation in the open areas (see Appendix iv). Although it would have been desirable to compare nitrogen-fixers with shrubs of approximately equal biomass, no other shrubs were in comparable abundance to *Shepherdia* and *Alnus* on sandy *Pinus/Populus* sites observed. Comparisons among leaves of different species presented no problems because many different shrub species occured on *Pinus/Populus* sites even though their abundance was limited.

Two sampling designs were implemented to accommodate various studies:

1. FIXED PLOT: Three representative areas from each shrub type were selected (ie. three groups of *Shepherdia* bushes, three groups of *Alnus* bushes, and three areas with only ericaceous shrubs). These areas within individual shrub types are referred to as 'shrubs'. This sampling design was used for soil information only. When soils were sampled, a specialized soil sampler consisting of a 15 cm shaft with a 5 cm diameter, was used. Seven cores from each 'shrub' were obtained at each sampling occasion. The cores were separated

into three depth layers:

0 to 5 cm

5 to 10 cm

surface organic layer (FH)

(leaf litter was not included)

Individual samples were pooled to produce one sample from each shrub at each depth.

2. RANDOM SELECTIONS: Samples were obtained from randomly selected shrubs or open areas. This design was used for all leaf and litter collections

All data were analyzed using the analysis of variance technique because of the sampling designs used. The significance level chosen for all analyses performed in Study II was an alpha level of .05. For a more detailed discussion of the rationale behind data analyses and the significance level selected, see the Methods section of Study I.

Detailed methods for individual studies follow in the order that the studies appear in the Results section.

LITTER ANALYSIS

Litter that was still in a definite form was collected early after snowmelt in April, 1980. Litter was collected from three open areas (ericaceous shrubs only), three *Shepherdia* areas, and three *Alnus* areas. The collections were air dried for three weeks and then separated into composition categories: woody matter; *Alnus* leaves; *Shepherdia* leaves; *Populus* leaves; *Pinus* leaves; other leaves; grass; cone fragments; and unidentifiable fragments. The percentage (by weight) that each litter type comprised in each sampling area was computed.

DESIGN: A random selections design was used for this study.

LFH DEPTH

Six cores from each of the three shrubs within the three shrub types were obtained and depth of LFH was measured for each (leaf litter was included with the FH for this analysis).

DESIGN: A fixed plot design was used for this study.

ANALYSIS OF GREEN LEAVES

Approximately ten leaves from the branch ends of 3 shrubs from each of the following species common to the study area were collected in June, 1980:

Arctostaphylos uva-ursi (L.) Spreng

Pinus banksiana Lamb.

Rosa acicularis Lindl.

Populus tremuloides Michx.

Salix bebbiana Sarg.

Amelanchier alnifolia Nutt.

Shepherdia

Alnus crispa

Petioles were removed and plants were oven-dried at 60° C for 24 hours (oven-dry weight). Micro Kejldahls (excluding nitrate) were performed using the procedure described in McKeague (1978).

DESIGN: A random selections design was used for this study.

ANALYSIS OF SENESCENT LEAVES

The methods for this study were identical to the methods used for the analysis of green leaves except that instead of *Arctostaphylos*, *Vaccinium myrtilloides* Michx. was analyzed (the same shrubs were not used for both studies).

C/N RATIOS IN THE FH LAYER

Three samples of the FH layer alone were obtained from each of the three shrubs within each of the three shrub types. Samples were air-dried and then oven-dried at 60°C for 24 hours in order to dry samples enough so that they could be ground. Samples were ground for about 2 minutes using a Tema swing mill until they were finely powdered. Carbon percent was determined by the Leko apparatus. Nitrogen concentrations were determined by micro Kejldahl (McKeague 1978).

DESIGN: A fixed plot design was used for this study.

CARBON AND NITROGEN IN MINERAL SOIL

Measures were obtained from air dry soils collected in June and July of 1980. Carbon was determined by the Walkley-Black method (McKeague 1978) and nitrogen was determined by micro Kejldahl (McKeague 1978). The two depths (0 to 5 cm) and (5 to 10 cm) were analyzed separately.

DESIGN: A fixed plot design was used for this experiment.

LITTER DECOMPOSITION

Alnus, *Shepherdia*, and *Populus* leaves were collected from beneath three *Alnus* shrubs, three *Shepherdia* shrubs, and three open areas respectively in April, 1980 (just after snowmelt). The leaves were air-dried and put into litter bags. Litter bags were constructed from fiberglass screening cut into 10 X 20 cm pieces. The bags were folded in half lengthwise and two open edges were folded over about 1 cm and stapled. (Conversions to oven-dry weights were

determined on a subsample of each leaf species). Bags were filled with 1 to 3 gms of leaves and stapled shut. Nine litter bags were constructed for each leaf species. Conversions to oven dry weights were determined on a subsample of each leaf species. Litter bags were buried at the end of May 1980 in the following experimental design:

Three litter bags from each leaf species were buried beneath one *Alnus* cluster, one *Shepherdia* cluster, and one open area. The bags were buried so that they were completely covered by the LFH layer.

At the end of the experiment, all litter was removed from the bags and weighed. Because of heavy sediment deposits on leaves, two bags from each species/shrub type combination were soaked in lukewarm tap water for about 5 minutes and rubbed gently to remove sand sediments. The sediment was filtered onto filter paper and oven dry weights of paper plus sediment were obtained after 24 hrs at 60°C. The sediment was then rinsed from the filter papers and papers were again oven dried. Percent weight loss was determined on all raw litter and adjusted weight loss was determined for two bags from each species/shrub combination. The washed litter gave the best results (in some cases, unwashed litter was actually recorded as gaining weight), so only washed litter was analyzed statistically.

DESIGN: A random selections design was used for this study.

INCUBATION STUDY

Two samples were taken from each soil depth: 0 to 5 cm; 5 to 10 cm; and FH for each individual shrub (54 samples). Approximately 14 g of mineral soil (o.d. weight) and 8 g of FH matter were placed into separate 250 ml plastic bottles capped with tin foil (samples were not dried before the incubations). Samples were abraded on a 1.18 mm mesh sieve to homogenize them. They were checked for moisture content every three days and moisture was adjusted to field capacity when it had dropped more than 10 percent. Samples were incubated for three weeks at 28° C and available nitrogen was extracted with 100 mls of 2 N KCl for each sample. Samples were shaken for 1 hr and filtered through Whatman #40 filter paper and steam distilled (see McKeague 1978). Percent water, percent carbon, and percent nitrogen were determined on pooled shrub type samples (*i.e.* the three shrubs within each shrub type were combined). Samples were pooled because of the extreme variability in fresh samples with respect to percent moisture and percent organic matter content. Incubation data were then adjusted so that data would reflect mineralization as though samples had contained equal carbon or equal nitrogen to better assess relative mineralization among shrub types. Statistical analyses were performed on each soil layer separately.

DESIGN: A fixed plot design was used for this study.

FIELD: AVAILABLE NITROGEN AND WATER

Samples were collected in the third week of the following months: May, June, July, and August and samples were analyzed within 24 hrs of collection. The extraction procedure for available nitrogen was the same as in the Incubation Study. Water percents were calculated on subsamples of collections oven dried for 24 hrs at 110° C. Moisture percent was calculated as: (moist weight - o.d. weight)/o.d. weight. Statistics from the three soil depths were analyzed separately.

DESIGN: A fixed plot design was used for this study.

VIII. STUDY II: RESULTS

A. LITTER ANALYSIS

Litter beneath nitrogen-fixers was concentrated within about a 1.5 m radius of the shrub bases. Means for the composition of litter beneath all shrubs and the composition of leaf litter beneath each individual shrub type are presented in Table XXIV.

Table XXIV. The general composition of litter beneath shrubs and the composition of leaf litter beneath individual shrub types.

Litter Category	Composition (% of total litter by weight)
woody	14
leaves	80
grass	3
unidentified fragments	3

	Leaf Litter Composition (% of leaf litter by weight)		
Leaf litter	OPEN	*SHEPHERDIA*	*ALNUS*
A. crispa	0	1	75
S. canadensis	0	33	0
P. tremuloides	57	37	12
P. banksiana	32	24	12
others	12	4	2

The main source of litter beneath shrubs was leaves and there were large differences among shrub types with respect to the concentrations of *A. crispa*, *S. canadensis*, *P. tremuloides*, and *P. banksiana* leaves. In the Open shrub type, leaves were exclusively *P. banksiana* and *P. tremuloides*. In the *Shepherdia* type there was a large concentration of *Shepherdia* leaves but *P.tremuloides* leaves predominated while beneath *Alnus*, only *Alnus* leaves were abundant.

B. LFH DEPTH

Alnus and *Shepherdia* shrub types had deeper LFH layers than the open type (see Table XXV).

Table XXV. The relative depth of the LFH layer beneath different shrub types.

ANOVA

SOURCE	df	MS	F	sig
shrub type	2	22.98	7.87	*
shrubs/type	6	2.92	4.56	*
error	45	.64		

MEANS AND TUKEY HSD (.05)

Open	1.1 cm	a
Shepherdia	2.7 cm	b
Alnus	3.2 cm	b

C. ANALYSIS OF GREEN LEAVES

The nitrogen concentrations of several plant species were measured in early summer. Means for these measures are presented in Table XXVI.

Table XXVI. The concentration of nitrogen in the leaves of several trees and shrubs in early summer.

SOURCE	df	MS	F	sig
species	7	1.597	19.72	*
error	15	.081		

MULTIPLE COMPARISONS (Tukey HSD at .05)

	% N	
Arctostaphylos uva-ursi	.9	c
Pinus banksiana	1.3	bc
Rosa acicularis	2.1	ab
Populus tremuloides	2.5	a
Salix bebbiana	2.6	a
Amelanchier alnifolia	2.7	a
Shepherdia canadensis	2.7	a
Alnus crispa	2.9	a

Arctostaphylos and *Pinus* leaves had low nitrogen concentrations relative to other species measured but most of the plant species analyzed had fairly high nitrogen concentrations and most were not significantly different from fixers with regard to nitrogen concentration.

D. ANALYSIS OF SENESCENT LEAVES

The nitrogen concentrations of senescent leaves are presented in Table XXVII.

Table XXVII. The concentration of nitrogen in the senescent leaves of several trees and shrubs collected in early autumn.

SOURCE	df	MS	F	sig
species	7	.6880	49.86	*
error	16	.0138		

MEANS AND MULTIPLE COMPARISONS (Tukey HSD at .05)

	% N	
Pinus banksiana	.5	b
Salix bebbiana	.7	b
Rosa acicularis	.7	b
Amelanchier alnifolia	.7	b
Vaccinium myrtilloides	.7	b
Populus tremuloides	.8	b
Shepherdia canadensis	1.6	a
Alnus crispa	1.8	a

Nitrogen-fixers had leaf nitrogen concentrations significantly higher than all other plant species measured: there was two to three times as much nitrogen contained in an equal weight of *Alnus* or *Shepherdia* leaves as there was in leaves of other selected species common in the Fort McMurray area.

E. C/N RATIOS IN THE FH LAYER

Differences among shrub types with respect to C/N ratios in the FH soil layer are presented in Table XXVIII.

Table XXVIII. Carbon/nitrogen ratios in the FH layer beneath different shrub types.

ANOVA

SOURCE	df	MS	F	sig
Shrub type	2	97.81	5.18	*
Shrubs/type	6	18.89	4.55	*
error	18	4.15		

MEAN C/N RATIOS

Open	25
Shepherdia	21
Alnus	18

Carbon/nitrogen ratios were significantly different among shrub types with the *Alnus* type displaying the narrowest C/N ratios and the Open type displaying the widest C/N ratios.

F. CARBON AND NITROGEN IN MINERAL SOIL

Variability in amounts of sand present within FH samples precluded analyses for percent carbon or percent nitrogen separately. In mineral soils, this variability was not a problem and ANOVAS of percent carbon and percent nitrogen characteristics as well as C/N ratios were run. Although none of the ANOVAS demonstrated significant differences at the .05 level, means were calculated (see Table XXIX).

Table XXIX. Carbon and nitrogen concentrations in mineral soil beneath different shrub types.

	MEANS		
	% Carbon	%NITROGEN	C/N
(0 to 5 cm)			
Open	.60	.03	24
Shepherdia	.85	.05	18
Alnus	.85	.05	19

	MEANS		
	% CARBON	%NITROGEN	C/N
(5 to 10 cm)			
Open	.22	.01	19
Shepherdia	.22	.02	15
Alnus	.32	.02	18

Even at very shallow soil depths, organic matter incorporation into mineral soil was minimal— most organic matter just remained on the soil surface as it decomposed.

G. LITTER DECOMPOSITION

The litter decomposition study was designed to observe the relative rates of decomposition among *Alnus*, *Shepherdia*, and *Populus* leaves. Means are presented in Table XXX.

Table XXX. The decomposition of *Shepherdia*, *Populus*, and *Alnus* leaves beneath different shrub types.

% weight loss means	LITTER TYPE		
SHRUB TYPE	*Shepherdia*	*Populus*	*Alnus*
Open	13	6	20
Shepherdia	27	11	25
Alnus	24	21	25

(The ANOVA is presented in Appendix iii, Table XI)

Leaves of the two nitrogen-fixers decomposed more readily than *Populus* leaves under each of the three shrub types.

H. INCUBATION STUDY

Table XXXI presents the means for the Incubation study. Data were adjusted to equal carbon concentrations within each soil depth to adjust for variable amounts of sands in samples. Shrub type was only significant with respect to mineralization in the FH soil depth.

Table XXXI. Mineralization of nitrogen from three soil layers beneath different shrub types over a three week incubation period (data adjusted to equal carbon concentrations).

MEANS		ppm (NH4+)-N	
		SOIL DEPTH	
SHRUB TYPE5	FH	0 to 5	5 to 10
Open	103	9.	0.3
Shepherdia	450	21.	2.
Alnus	500	19.	6.

(ANOVA for the FH layer is included in Appendix iii, Table XII)

Virtually no nitrate was detected in incubation samples.

Data from the Incubation Study were also adjusted to equal nitrogen concentrations to better assess rates of nitrogen mineralization (see Table XXXII). Again, significant differences with respect to shrub type were only found in the FH layer.

Table XXXII. Mineralization of nitrogen from three soil layers beneath different shrub types over a three week incubation period (data adjusted to equal nitrogen concentrations).

MEANS	ppm (NH4+)-N SOIL DEPTH		
SHRUB TYPE	FH	0 to 5	5 to 10
Open	122.0	11.0	1.0
Shepherdia	450.0	21.0	2.0
Alnus	500.0	18.0	5.0

(The ANOVA for the FH layer is included in Appendix iii, Table XIII.)

I. FIELD: AVAILABLE NITROGEN AND WATER

The ANOVA of available nitrogen obtained from field samples did not demonstrate any significant differences among shrub type FH layers although the *Alnus* mean was considerably higher than the *Shepherdia* or Open area mean. (Only the FH layer was statistically analyzed because of the high frequency of 0 values at other depths.) Available nitrogen varied significantly among sampling times but this variability appeared to be associated with normal field variability rather than depicting a seasonal trend. Means and ranges for available nitrogen in different shrub types at different depths computed over the whole sampling season are presented in Table XXXIII. Means and standard errors for shrub types at different sampling times are included for the FH layer in the same table.

Table XXXIII. Available nitrogen present in field soil samples collected beneath different shrub types at different depths at different sampling times.

Means and ranges ppm (NH_4^+)-N

	SOIL DEPTH		
SHRUB TYPE	FH	0 to 5	5 to 10
Open	6(0-52)	0(0)	0(0)
Shepherdia	14(2-53)	.2(0-1)	0(0)
Alnus	26(2-98)	1.0(0-1)	0(0-1)

Table XXXIII. Cont'd

Means and standard deviations ppm (NH4+)-N
(for the FH layer only)

SHRUB TYPE	SAMPLING TIME MAY		JUNE		JULY		AUG	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Open	1	1	18	29	7	4	0	
Shepherdia	6	5	32	18	17	5	3	2
ALnus	8	3	65	29	24	13	6	3

At any one sampling time, average available nitrogen in the *Alnus* FH was always higher than average available nitrogen in *Shepherdia* or open area FH.

Soil moisture differences among shrub types at any depth were not statistically significant at the .05 level using an ANOVA test. Sampling time was significant, but again this did not appear to be due to any seasonal trend. Means and ranges for each depth over the whole sampling period are included in Table XXXIV. Means and standard errors for each sampling time are presented for the FH layer only at the bottom of the same table.

Table XXXIV. The proportion of moisture in field soil samples collected beneath different shrub types at different depths at different sampling times.

Means and ranges (% H2O)

SHRUB TYPE	SOIL DEPTH FH	0 to 5	5 to 10
Open	65 (14-184)	7 (1-14)	3 (1-9)
Shepherdia	66 (16-171)	7 (1-16)	4 (1-7)
Alnus	69 (25-155)	8 (2-19)	4 (1-10)

Means and standard deviations (% H2O)

SHRUB TYPE	SAMPLING TIME MAY		JUNE		JULY		AUG	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Open	155	26	19	2	15	1	71	25
Shepherdia	114	49	33	6	23	7	93	3
Alnus	88	21	45	18	28	5	117	35

Except for the May estimate, the FH layer was moister under *Alnus* than other shrub types. The May sample contained a relatively high proportion of sand and this may have been responsible for the low moisture estimate.

IX. STUDY II: DISCUSSION

The ages of *Shepherdia* and *Alnus* on the study site ranged from about 10 to about 25 years (based on the number of rings at stem bases), and it is quite likely that many of the shrubs investigated were not fixing nitrogen at the time measurements were made. However, it was assumed that at least some shrubs had been fixing nitrogen at some point during their colonization of the study site, and that observations made on nitrogen concentrations and decomposition rates of litter would hold for *Shepherdia* and *Alnus* whether the shrubs were actively fixing nitrogen or not.

There is a paucity of literature concerning the composition of litter beneath nitrogen-fixing shrubs. It must be generally assumed that litter from beneath a particular shrub consists mainly of contributions from that shrub. The investigation conducted in this study shows that this is not always true (see Table XXIV). For *Alnus*, a taller shrub with more leaf biomass than *Shepherdia*, the assumption held quite well but *Shepherdia* litter was 'diluted' with leaf litter from adjacent non-fixing species. Nitrogen-fixer leaf litter only accumulated at the bases of the shrubs from which litter fell and almost all litter was found within about a 1.5 m radius of shrub bases. Distribution of *Alnus* and *Shepherdia* litter was limited by the short, branchy nature of the shrubs which tends to 'trap' their own litter. If nitrogen transfer to non-fixing

shrubs is a function of distance to fixer residues, then the range of nitrogen influence that *Shepherdia* has at the soil surface is limited to a very narrow area around the shrub base.

Root distributions and activities beneath the soil surface were not investigated directly and several authors have alluded to the importance of root decomposition or nitrogen secretions into the rhizosphere as a means of nitrogen transfer (Gadgil 1971; Virtanen 1957; Virtanen and Miettinen 1963; Zavitkovsky and Newton 1968). However, observations during nodule excavations indicated that roots of *Shepherdia* are not extensive horizontally or vertically beneath the soil surface and again nitrogen accumulation beneath the soil surface would be localized to shrub bases.

Total annual litter amounts were not measured directly but LFH depth served as an indication of the relative amounts of litter added to shrub types annually. LFH depths were greater beneath *Alnus* and *Shepherdia* than in the open (see Table XXV). This was expected because there was much more leaf biomass on *Shepherdia* and *Alnus* shrubs than there was over open areas. However, the depths of LFH measured do not likely correspond directly to amounts of litter inputs. Several researchers have found that accumulation of LFH beneath nitrogen fixers can actually be less than accumulations beneath non-fixing shrubs which release similar amounts of litter (Daly 1966; Newton *et al.* 1968; Tarrant and Miller 1963; Voight and Steucek 1969).

Apparently, litter from nitrogen-fixers may encourage more rapid decomposition and this is responsible for lower rates of accumulation. Thus, although the depth of *Alnus* LFH was three times that of LFH in the Open area, litter inputs may have been more than three times as great beneath *Alnus*.

Analyses of leaf nitrogen concentrations demonstrated that nitrogen-fixers translocate less of their leaf-nitrogen back into plant parts before senescence than non-fixers (see Tables XXIV and XXV). Although these plants have the ability to fix nitrogen, the fixation process requires energy and it is not clear why they should not retain more nitrogen. Perhaps more of the nitrogen in fixer leaves is associated with structural compounds which are not easily mobile. The concentrations of nitrogen in senescing leaves of *Alnus* and *Shepherdia* were very high (1.8% and 1.6% respectively). These estimates compared favourably with estimates of leaf nitrogen concentrations in other actinomycete-nodulated plants. Cromack *et al.* (1979) found nitrogen concentrations of 2.1% and .8% in senescent leaves of *Alnus rubra* and *Ceanothus velutinus* respectively. As a general rule, substrates containing more than 1.8 percent nitrogen can undergo net mineralization as soon as the substrate begins decomposition. (Alexander 1977).

As litter decomposition proceeds, carbon/nitrogen ratios decrease (Alexander 1977). Carbon/nitrogen ratios of the FH soil layer reflected differences in the nitrogen concentrations of litter inputs but the mean Open area

carbon/nitrogen ratio was only 4/3 as high as the *Alnus* C/N ratio while the bulk of litter undergoing decomposition initially had a carbon/nitrogen ratio over twice as great as *Alnus* (see Table XXVIII). Carbon/nitrogen ratios, then, were not an extremely sensitive index of nitrogen accretion beneath nitrogen-fixing shrubs.

Carbon and nitrogen concentrations of mineral soil reflected the slow incorporation of organic matter into mineral soil on the study site. Although decomposition of the litter occurred, residues were not mixed into mineral soil, they just accumulated on the forest floor. The FH soil layer contained virtually 100 percent organic matter and over 1 percent nitrogen in each shrub type, and yet mineral soils only 5 cm beneath this layer contained less than 1 percent carbon and less than .05 percent nitrogen (see Table XXIX). Slow incorporation of organic residues may have been a function of the study site's climate which restricts biological activity for a good part of the year (see the climatic characteristics of the study site described in the Methods section of Study II).

Leaf decomposition over time was measured as an index of how readily nitrogen can be released from leaves. Even if nitrogen-fixer leaves have high nitrogen concentrations, mineralization will not proceed unless leaves can be readily degraded by soil organisms. Decomposition differences were significant with respect to species: *Alnus* and *Shepherdia* leaves decomposed more readily than *Populus* leaves under

three different shrub covers (see Table XXX). Decomposition estimates for senescent *Ceanothus velutinus* and *Alnus rubra* leaves indicated that about 50 percent of leaf biomass is decomposed after 1 year (Cromack *et al.* 1979). Estimates obtained for *Shepherdia* and *Alnus* were about half this rate but decomposition was only measured over a three month period and the other estimates were obtained from studies in the Pacific Northwest where decomposition likely proceeds more rapidly. Leaf decomposition was also affected by shrub cover. For each leaf species analyzed, decomposition was faster beneath *Alnus* and *Shepherdia* than in the open. Moisture differences among shrub types may have been important although moisture within any one shrub type was variable and moisture differences among shrub types were not significant at the .05 level. A shrub cover may tend to shelter soils from evaporation losses, and the greater litter depths beneath shrubs (see Table XXV) may also tend to reduce evaporation losses. Soil-organism populations beneath the three shrub types may have varied and this may have affected decomposition rates beneath different shrub covers as well.

Incubation studies showed that organic residues beneath *Alnus* and *Shepherdia* had the potential to mineralize large amounts of nitrogen (see Tables XXXI and XXXII). Incubation studies performed by Bollen and Lu (1968) document mineralization rates of about 200 ppm of available nitrogen over a one month period in the F layer beneath *Alnus rubra*.

Levels obtained with *Shepherdia* and *Alnus crispa* FH were closer to 500 ppm, but greater mineralization of *Alnus crispa* and *Shepherdia* residues than *Alnus rubra* residues was probably more indicative of the pre-histories of residues than of the quality of residues. *Alnus rubra*, collected from the Pacific Northwest, would likely have undergone more decomposition than FH collected from beneath *Shepherdia* and *Alnus crispa* for reasons already mentioned. *Shepherdia* and *Alnus crispa*, then, may have had more readily oxidizable carbon in their residues which would have supported rapid mineralization over some period before readily available carbon was exhausted. This hypothesis is supported by the lower C/N ratios in the F layers examined by Bollen and Lu (1968) (18/1 for *Alnus crispa* vs. 15/1 for *Alnus rubra*). Mineralization in FH residues collected from open areas was considerably less than mineralization in *Shepherdia* or *Alnus crispa* residues. However, because of the lower annual input of leaf litter into open areas, a greater proportion of the residue was probably more highly degraded. Thus, nitrogen mineralization in the FH layer of the Open shrub type was not likely representative of nitrogen release from residues of non-fixing shrubs with litter quantities similar to *Alnus* or *Shepherdia*. It would have been best to compare residues beneath nitrogen-fixers with residues beneath similar-sized shrubs but no larger shrubs were in comparable abundance to *Shepherdia* on or near the study site.

Trends indicated that, under field conditions, more

nitrogen was mineralized beneath *Alnus* than *Shepherdia* or open shrub types (see Table XXXIII). *Shepherdia* was expected to have field mineralization comparable to *Alnus* considering its high incubation mineralization but this was not the case. Perhaps in the field where fresher residues were present (the incubation study examined only the FH layer), the abundant *Populus* litter (high carbon/nitrogen) beneath *Shepherdia* tended to bring about immobilization of nitrogen. Soil moisture regimes may have been more conducive to decomposition and to the mineralization activities of microbes beneath *Alnus*, but differences in moisture levels beneath *Alnus* and *Shepherdia* were not large (see Table XXXIV).

Mineralization and moisture means among shrub types were distinct although statistically significant differences were not demonstrated. The major component of variability in assessing soil nitrogen or moisture in soils beneath a particular shrub species is variability among shrubs within the species. For most analyses, three shrubs per shrub type was an adequate sample size for obtaining statistically significant differences, but soil variability was so great with respect to mineralization and moisture that more shrub collections would be required to statistically verify any differences among shrub type means. The recognition of this variability is important because it demonstrates that, under diverse field conditions, a particular shrub can affect soil quite differently from what is generally expected.

X. CONCLUSIONS

A. STUDY I

Regarding the effects of artificial inoculation on nodule development and seedling survival:

1. Nodules can be induced in *Shepherdia* using an artificial inoculum and growing seedlings under operational greenhouse conditions (*i.e.* in containerized peat media).
2. Although data must still be collected to determine if nodulation enhances outplanting survival, preliminary evidence suggests that seedlings inoculated before outplanting produce more root growth than uninoculated seedlings and this could be a survival advantage.

Other conclusions which can be drawn from the Study are:

1. The inoculum or the inoculation procedure used for inoculating *Shepherdia* seedlings should be improved to increase the occurrence of nodulated seedlings.
2. Nodules can develop in seedlings provided with nitrogen levels adequate for the production of seedlings as large as seedlings presently being outplanted.

3. The per nodule fixation rates of *Shepherdia* are within the range of other actinomycete-nodulated nitrogen-fixers.
4. Inoculated seedlings, through nodulation, contain more nitrogen in their tissues and this nitrogen is used to increase seedling growth. Hence, nodulated seedlings tend to be larger than uninoculated seedlings both before and after outplanting.
5. Native inoculum can nodulate plants after outplanting and relative advantages of pre-nodulated seedlings, in terms of growth and survival after outplanting, may be reduced as native-inoculum nodules develop. Data collections over the next two years will examine this effect.

B. STUDY II

Conclusions regarding *Shepherdia*'s contributions to soil nitrogen are as follows:

1. Total nitrogen does accumulate in soil beneath *Shepherdia* probably as a result of accumulations of *Shepherdia's* nitrogen-rich leaf litter.
2. Nitrogen only accumulates in about a 1.5 m radius beneath *Shepherdia's* base, so any nitrogen benefits to other plants would have to come from plants growing very near to *Shepherdia*.

3. Organic residues of *Shepherdia* tend to accumulate on the surface of mineral soil rather than becoming incorporated thus, nitrogen release to adjacent plants is limited along a vertical as well as a horizontal plane.
4. Nitrogen-rich organic residues beneath *Shepherdia* mineralize nitrogen readily under laboratory incubation conditions (*i.e.* moisture near field capacity and temperatures about 28°C).

Other conclusions which may be drawn from the study are as follows:

1. The leaves of *Shepherdia* and *Alnus crispa* appear to translocate less of their leaf nitrogen back into stems and roots than several other shrub species. The concentration of nitrogen in the senescent leaves of *Shepherdia* and *Alnus* is about two to three times as great as the concentration of nitrogen in the leaves of several non-fixing shrubs common in the Fort McMurray area.
2. The leaves of *Shepherdia* and *Alnus crispa* appear to decompose more readily than the leaves of *Populus tremuloides*.

3. *Shepherdia* compares favourably to *Alnus crispa* with respect to the quality of nitrogen residues. However, *Alnus* is a larger shrub and the quantity of its nitrogen-rich residues accumulating in soils would be greater than for *Shepherdia*.

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XII. APPENDIX 1: FERTILIZER SOURCES

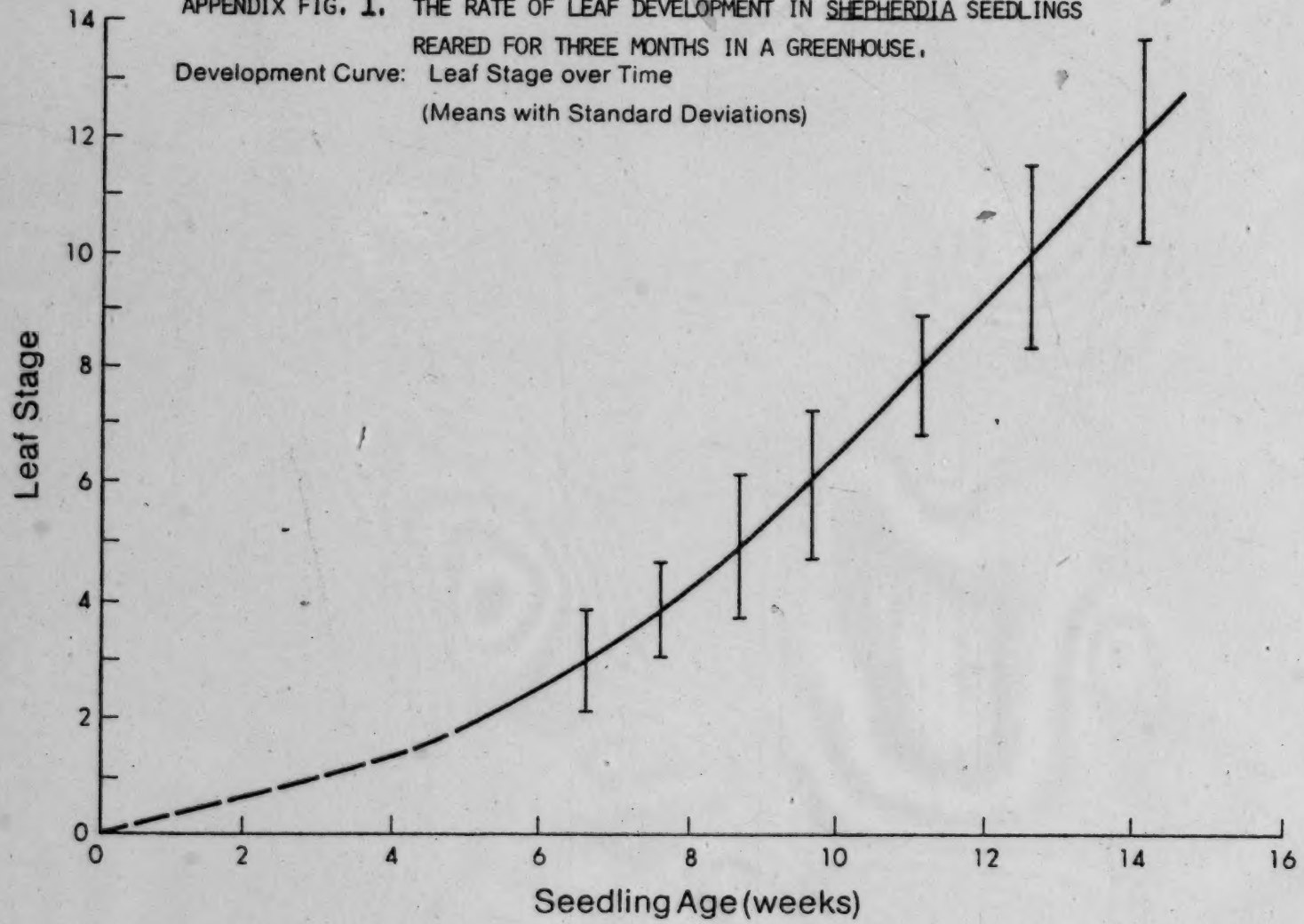
N	NH4NO3
P	NaH2PO4
K	KCl
S	Na2SO4
B	H3BO3
Mn	MnCl2*4H2O
Zn	ZnSO4*7H2O
Mo	HMoO4
Cu	CuSo4*5H2O

XIII. APPENDIX ii: GROWTH CURVES

APPENDIX FIG. 1. THE RATE OF LEAF DEVELOPMENT IN SHEPHERDIA SEEDLINGS REARED FOR THREE MONTHS IN A GREENHOUSE.

Development Curve: Leaf Stage over Time

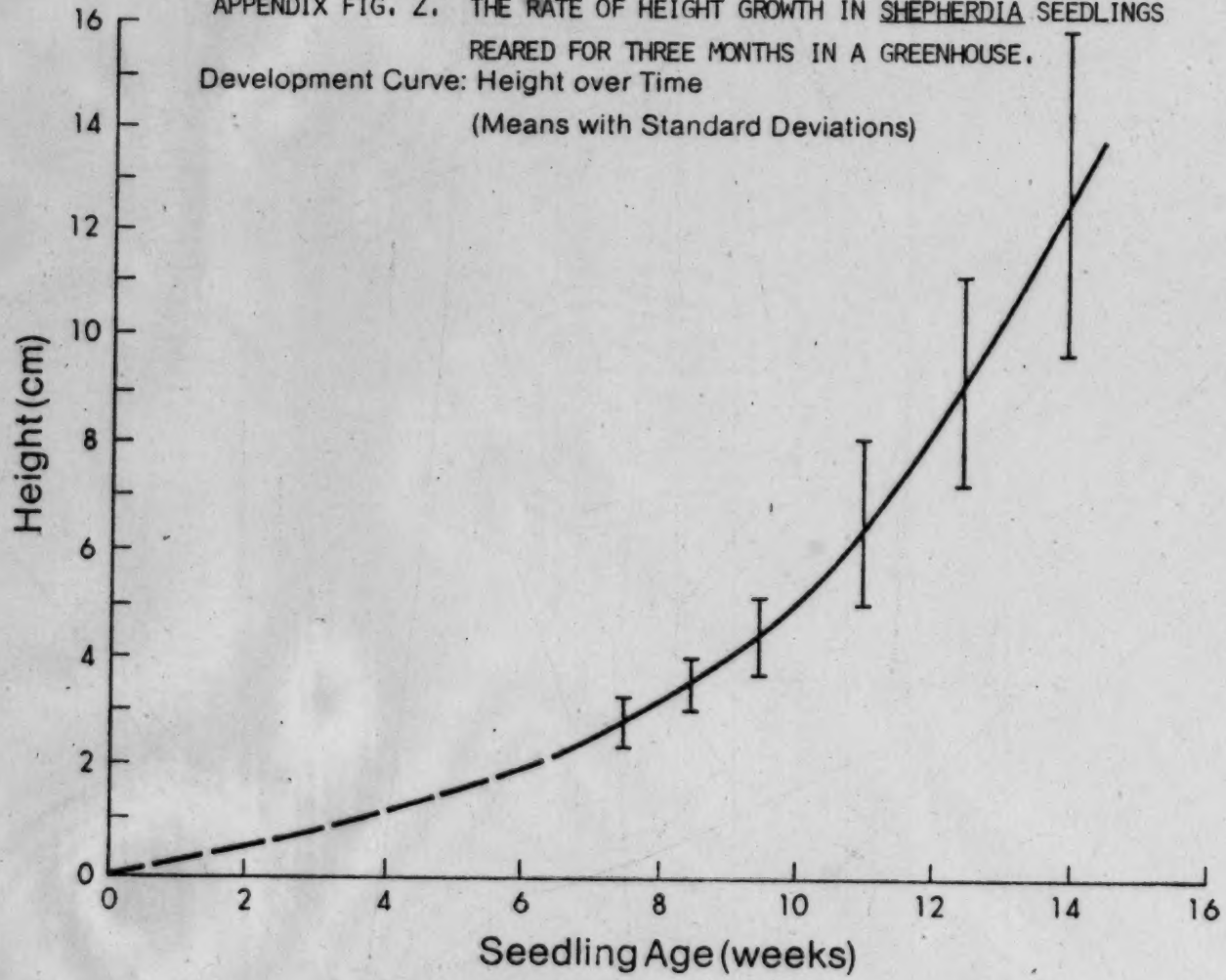
(Means with Standard Deviations)



APPENDIX FIG. 2. THE RATE OF HEIGHT GROWTH IN SHEPHERDIA SEEDLINGS REARED FOR THREE MONTHS IN A GREENHOUSE.

Development Curve: Height over Time

(Means with Standard Deviations)



XIV. APPENDIX iii: APPENDIX TABLES

Table I. ANOVA for Study I, text Table VIII.

ANOVA for shoot weights

SOURCE	df	EMS	F	sig
Peat	4	.241	13.35	*
Nitrogen	2	8.6223	484.13	*
P X N	8	.1897	10.65	*
error	105	.0178		

ANOVA for root weights

SOURCE	df	EMS	F	sig
Peat	4	.1076	20.23	*
Nitrogen	2	1.2047	226.45	*
P X N	8	.0913	17.16	*
error	105	.0178		

Table II. ANOVA for Study I, text Table IX.

SOURCE	df	MS	F	sig
Block	8	1.14		
Nitrogen	2	.13	.24	NS
Inoculum	1	2.24	4.21	*
Nit X Inoc	2	.02	.03	NS
error	40	.53		

Table III. ANOVA for Study I, text Table XI.

ANOVA for nodule number

SOURCE	df	MS	F	sig
Nitrogen	2	2.93	.16	NS
Inoculum	1	260.5	13.97	*
N X I	2	15.56	.83	NS
error	30	18.65		

ANOVA for Nodule biomass

SOURCE	df	MS	F	sig
Nitrogen	2	144.07	3.98	*
Inoculum	1	408.91	11.31	*
N X I	2	9.61	.27	NS
error	30	36.17		

Table IV. ANOVA for Study I, text Table XII.

SOURCE	df	MS	F	sig
nitrogen	2	43416.75	1.5	NS
inoculum	1	46614.68	1.6	NS
nit X inoc	2	42824.44	1.5	NS
error	30	28887.51		

Table V. ANOVA for Study I, text Table XVI.

ANOVA for shoot weight

SOURCE	df	MS	F	sig
Blocks	8	.067		
Inoculum	2	.206	7.6	*
Nitrogen	2	7.178	265.9	*
N X I	4	.041	1.5	NS
error	64	.027		

ANOVA for root weight

SOURCE	df	MS	F	sig
Blocks	8	.004		
Inoculum	2	.007	1.9	NS
Nitrogen	2	.736	204.4	*
N X I	4	.004	1.1	NS
error	64	.0036		

Table VI. Regression statistics for the relationship between nodule biomass and shoot and root weights after the growth room rearing period.

shoot weight with nodule biomass

	r2	b	(s.e. b)	F	sig
N-	.96	.02	.001	246.8	*
N+	.93	.02	.001	268.1	*
Full N	.24	.01	.006	4.1	*

root weight with nodule biomass

	r2	b	(s.e. b)	F	sig
N-	.93	.004	.0003	145.3	*
N+	.57	.002	.0004	25.0	*
Full N	.28	.006	.003	5.1	*

Table VII: ANOVA for Study I, text Table XVIII.

ANOVA for nodule number

ANOVA: SOURCE	df	MS	F	sig
R	2	446.99	.79	NS
R/O	6	564.52		
N	2	21051.25	9.49	*
I	2	6060.37	2.73	NS
NI	4	1705.59	.77	NS
ON	4	484.38	.22	NS
OI	4	17.08	.01	NS
error	51	2217.98		
error (s)	55	238.54		

ANOVA for nodule biomass

SOURCE	df	MS	F	sig
O	2	1153.73	1.46	NS
R/O	6	792.42		
N	2	23784.84	9.72	*
I	2	8073.98	3.30	*
NI	4	2567.81	1.05	NS
ON	4	997.86	.41	NS
OI	4	768.30	.31	NS
error	51	2446.23		
error (s)	55	35.59		

Table VIII. ANOVA for Study I, text Table XX.

ANOVA for shoot weight

SOURCE	df	MS	F	sig
O	2	.675	4.27	NS
R/O	6	.16		
N	2	9.90	104.26	*
I	2	.49	5.16	*
NXI	4	.04	.42	NS
ON	4	.41	4.32	*
OI	4	.03	.26	NS
error	51	.095		
error(s)	55	.049		

ANOVA for root weight

SOURCE	df	MS	F	sig
O	2	.183	3.10	NS
R/O	6	.059		
N	2	5.961	96.14	*
I	2	.220	3.55	*
NXI	4	.081	1.29	NS
ON	4	.138	2.23	NS
OI	4	.065	1.05	NS
error	51	.062		
error (s)	55	.023		

Table IX. Regression statistics for the relationship between nodule biomass and shoot and root weights after outplanting.

shoot weight with nodule biomass

	r2	b	(s.e. b)	F	sig
N-	.73	.01	.001	88.9	*
N+	.61	.01	.001	75.5	*
Full N	.35	.01	.001	23.89	*

root weight with nodule biomass

	r2	b	(s.e. b)	F	sig
N-	.87	.01	.001	214.5	*
N+	.70	.01	.001	110.0	*
Full N	.38	.005	.001	26.86	*

Table X. ANOVA for Study I, text Table XXII.

SOURCE	df	MS	F	sig
O	2	.0586		NS
R/O	6	.1482		
N	2	.3086	5.48	*
I	2	.5586	9.92	*
NXI	4	.2346	4.16	*
error	64	.0563		

Table XI. ANOVA for Study II, text Table XXX.

SOURCE	df	MS	F	sig
shrub type	2	172.9	3.6	*
litter type	2	188.8	4.0	*
shrub X litter	4	34.1	.7	NS
error	9	47.4		

Table XII. ANOVA for Study II, text Table XXXI.

SOURCE	DF	MS	F	SIG
Type	(2)	280171	7.2	*
s/Type	(6)	38884	4.5	*
error	(9)	8671		

Table XIII. ANOVA for Study II, text Table XXXII.

SOURCE	DF	MS	F	SIG
Type	(2)	252869	6.4	*
s/Type	(6)	39619	4.5	*
error	(9)	8872		

XV. APPENDIX iv: VEGETATION OF THE STUDY SITE

	% aerial cover		
	OPEN	*SHEPHERDIA*	*ALNUS*
Amelanchier alnifolia	1	+	+
Lonicera dioica	1	+	+
Pinus banksiana	1	0	0
Populus tremuloides	2	2	+
Rosa acicularis	2	1	1
Arctostaphylos uva-ursi	41	19	6
Vaccinium myrtilloides	16	6	13
Vaccinium vitis-idaea	1	1	1
Achillea millefolium	0	0	+
Anemone multifida	+	+	+
Commandra pallida	2	1	+
Cornus canadensis	0	+	0
Epilobium angustifolium	0	0	+
Fragaria virginiana	+	+	+
Galium boreale	1	2	+
Grass spp.	7	8	11
Lathyrus ochroleucus	1	+	0
Lycopodium complanatum	6	3	7
Maianthemum canadense	1	5	3
Pyrola secunda	0	+	1
Moss spp.	0	+	1
Cladina spp.	17	4	1
exposed litter	2	12	14
decaying wood	5	1	1

XVI. APPENDIX v: FERTILIZER EXPERIMENT TECHNICAL DETAILS

1. Duration:

   September 24,1979 to January 8,1980

2. Seedlot:

   Same as the Preliminary Growth Experiment

3. Nutrients:

   Total nutrients supplied (excepting the manipulated nutrient) were as follows: (expressed as Kg/Ha equivalents based on container surface area).

Nitrogen experiment:

nutrient	mg/seedling	kg/ha
P	49	340
K	53	365
S	16	110

Phosphorous experiment:

nutrient	mg/seedling	kg/ha
N	49	340
K	46	320
S	16	110

Potassium experiment:

nutrient	mg/seedling	kg/ha
N	49	340
P	49	340
S	16	110

Sulphur experiment:

nutrient	mg/seedling	kg/ha
N	49	340
P	49	340
K	46	320

Iron chelate and micro nutrients were applied at the same rates in all experiments as follows:

nutrient	dosage (micro g)
B	117
Mn	124
Zn	14
Mo	4
Cu	5
Co	12
Fe chelate	.024 g/seedling

4. Experimental design:

   Four separate two factor factorial experiments arranged as randomized complete blocks (one experiment for each macro nutrient)

5. Treatments:

   a) 6 levels of the experimental nutrient: prepared to represent approximately 150%, 100% ,75% ,50 %,25 %,and 0% of dosages administered in the Initial Experiment. (expressed as Kg/Ha equivalents based on container surface area)

	per seedling application rates			
	N		P	
LEVEL	mg	kg/ha	mg	kg/ha
150%	77	530	80	550
100%	51	350	52	360
75%	38	260	39	270
50%	25	170	26	180
25%	13	87	13	90
0	0	0	0	0

LEVEL	K mg	K kg/ha	S mg	S kg/ha
150%	74	510	25	170
100%	49	335	16	110
75%	36	250	12	85
50%	24	165	8	55
25%	12	80	4	28
0	0	0	0	0

b). 2 media:

(P/V) peat/vermiculite (2:1)

(V) vermiculite alone

Each experiment was arranged into two adjacent blocks to adjust for variability in growth conditions along the greenhouse bench.

6. Experimental procedures:

Approximately equal amounts of each fertilizer nutrient were supplied to seedlings at each application.

7. Experiment dimensions:

1 container row per unique treatment combination (randomized) with 4 seedlings (repeats) within each container row.

(NOTE: seedlings were planted in every planting cavity with alternate rows left empty)

(6 nurient levels X 2 media X 4 repeats X 2 blocks)= 96 seedlings per experiment

8. analysis:

-repeats were pooled providing one mean observation per unique treatment combination

-data were analyzed using the APL programs AOV5 for ANOVAS and MEANS to compute means.

RESULTS

NOTE: due to high mortality in this experiment, data for the peat/vermiculite and the vermiculite medium were pooled.

Nitrogen Experiment Means

A. Roots (error mean square .002)

N level (kg/ha)	wt (gms)
0	.014
87	.088
170	.173
260	.165
350	.123
530	.210

B. Shoots (error mean square .027)

N level (kg/ha)	wt (gms)
0	.021
87	.233
170	.524
260	.536
350	.440
530	.708

At levels of nitrogen greater than 170 kg/ha, neither shoot nor root weight increased substantially. However, seedlings appeared slightly chlorotic at the 170 kg/ha level, so it was decided to set the nitrogen level for future experiments at about 260 kg/ha.

Phosphorous Experiment Means

A. Roots (error mean square .001)

P level (kg/ha)	wt (gms)
0	.018
90	.174
180	.135
270	.171
360	.157
550	.210

B. Shoots (error mean square .017)

P level (kg/ha)	wt (gms)
0	.027
90	.654
180	.567
270	.625
360	.629
550	.673

At 90 kg/ha plants were not showing significant weight gains with added phosphorous. Thus a phosphorous level of about 90 kg/ha was selected for later experiments.

Neither the Potassium Experiment nor the Sulphur experiment seedlings demonstrated any significant response to varying nutrient levels.